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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : C12N 15/82, A01H 5/00

A1

(11) International Publication Number: WO 00/63398

(43) International Publication Date: 26 October 2000 (26.10.00)

(21) International Application Number: PCT/EP00/04177

(22) International Filing Date: 19 April 2000 (19.04.00)

(30) Priority Data:
99420097.0 19 April 1999 (19.04.99) EP

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(81) Designated States: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
*With international search report.*

(54) Title: PLANT TRANSFORMATION METHOD

(57) Abstract

A transformation method comprising inoculation and co–cultivation of a target tissue, from a target plant, with *Agrobacterium*, at a time when the target tissue is in its natural plant environment, followed by generation of a transgenic plant via dedifferentiation and regeneration of the target tissue.

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# PLANT TRANSFORMATION METHOD.

The present invention provides a method for the *Agrobacterium*-mediated transformation of plants, in particular monocotyledonous plants.

The invention is in the field of plant transformation, in particular cereal transformation, specifically in the use of *Agrobacterium tumefaciens* or any other *Agrobacterium* species (hereinafter referred to as « *Agrobacterium*»). Until recently, only direct transformation methods could be used to produce transgenic cereal plants. Bombardment using a particle gun is the most widely accepted method to this end. More recently, reports have appeared in the literature showing that some of the cereals can be genetically modified using *Agrobacterium* (Hiei *et al*, Plant Mol. Biol. (1997) 35:205-218); Ishida *et al*, Nature Biotechnol. (1996) 14:745-750; Cheng *et al*, Plant Physiol. (1997) 115:971-980; Tingay *et al.*, The Plant Journal, 11:1369-1376 (1997)).

Transformation efficiencies reported in the literature show wide variability for different cereals. Typically, low figures have been quoted for maize (Ishida 1996), with a system that is highly genotype dependent. With rice, low efficiencies for transformation have also been reported, and particularly low levels have been shown for wheat. In all of these systems, *Agrobacterium* is applied *in vitro*, to isolated tissue that is either in the process of de-differentiation or is already de-differentiated.

As described above, systems for *Agrobacterium*-mediated transformation of cereals have been reported in rice (Hiei, 1997), maize (Ishida, 1996), wheat (Cheng, 1997) and barley (Tingay, 1997). A common feature of these methods is that explants, preferably immature embryos or embryogenic calli derived therefrom, are isolated from a donor plant and inoculated with *Agrobacterium in vitro.*

Hess and coworkers (Plant Science 72: 233-244, 1990) attempted transformation of wheat by pipetting *Agrobacterium* into spikelets of wheat. The authors objective in this report was to achieve gene transfer by transformation of pollen and to subsequently recover transformed seed following normal fertilization. Removal of tissue from the inoculated spikelet for subsequent selection and regeneration in culture was not attempted or suggested.

Other workers have reported the *Agrobacterium*-mediated transformation of maize and rice by inoculation of shoot apices (Gould J (1991) Plant Physiol. 95: 426-434; Park SH (1996) Plant Molecular Biology 32: 1135-1158). Once again, this was with the object of transforming the germ line and thus recovering transformed seed. This pathway of regeneration is distinct from that employed in the method of our invention: in fact, a specified aim of these methods is to avoid any method of plant regeneration going through dedifferentiation of tissue and adventitious regeneration.

U.S. Patents 5,177,010 and 5,187,073 (Goldman, *et al*) disclose a method for transforming corn and Gramineae respectively, comprising wounding newly emerged seedlings and inoculating with *Agrobacterium*. Once again, the objective of this method is to transform germ line cells in the seedling that will subsequently give rise to reproductive organs in the mature plant and thereby recover transformed pollen from the plant.

Another process that has been studied by those trying to develop cereal transformation is agroinfection. U.S. Patent No. 5,569,597 (Grimsley, *et al*) discloses a method of introducing viral DNA into plants using *Agrobacterium*. Following inoculation of maize seedlings with *Agrobacterium* having DNA from maize streak virus inserted in its T-DNA, the inventors observed the appearance of disease symptoms, indicating proliferation of virus in plant cells. The *Agrobacterium* therefore acts as a vehicle to introduce the viral DNA into the plant, after which the virus is able to cause a systemic infection. However, there is no evidence that agroinfection results in plant transformation *i.e.* transfer of viral DNA to the plant genome. In so far as the patent considers transformation it is, once again, with a view to targeting meristematic tissues in order to achieve transformation of germ cells.

In this novel method, the targeted tissue is inoculated and co-cultivated with *Agrobacterium* when the target tissue is within its natural plant environment. In this way, the target tissue is still developing along normal physiological and temporal pathways. The target tissue is then removed from its normal environment and directed along a pathway of dedifferentiation and regeneration to form a transgenic plant. Advantageously, the transgenic plant is a fertile transgenic plant.

In the present invention, the term «within its natural plant environment» includes all conditions where the target tissue is able to develop along substantially normal physiological and temporal pathways. Such conditions include the target tissues being *in vivo*, the target tissue still being within, on or attached to the plant (for example the target tissue being an embryo within a seed on a cut tiller), or target tissue which is still in the same cellular environment that it would be if it were still on the plant (for example the target tissue being an embryo within an isolated seed, or part of an isolated seed). Other examples include immature inflorescence still within the leaf sheath or at least still attached to the plant and an immature anther while still in the unopened flower bud.

Dedifferentiation means cell clusters, such as callus, that show unorganized growth.

In addition to the target tissue being in an environment equal to that on the plant, the *Agrobacterium* is in an environment that is more analogous to the bacteria's natural environment. Accordingly the *Agrobacterium* is likely to act more efficiently in its transformation of the target tissue than when it is directed to an isolated tissue in a petri dish, as in the art.

One consequence of these two factors is the opportunity to obtain a higher transformation efficiency of the desired transgene to the target tissues and thus a higher transformation efficiency for the production of transgenic plants.

One of the primary steps involved in most transformation protocols involves wounding the target tissue. With *Agrobacterium*, this can be for two reasons - to expose the cells thought to be responsive to transformation and that are capable of regeneration, (particularly for Gramineous species) and to induce the *Agrobacterium* to transfer its T-DNA. One published method for wheat that does not involve wounding still involves the use of a wetting agent (Silwet or pluronic acid) or vacuum infiltration (WO 97/48814). All these processes involve some inherent damage to the tissue, and an associated reduction in regenerative capability.

In a preferred embodiment of the present invention, wounding of the target cells in the target tissue is kept to a minimum or totally excluded - although a wetter may be used, it is not essential. Some gross damage of tissue may occur during the delivery

procedure, but even then the vast majority of regenerable cells that are subsequently targeted by the *Agrobacterium* remain undamaged and their regenerative capacity is unaffected.

According to the present invention, inoculation of *Agrobacterium* is preferably done by the application of an *Agrobacterium* suspension to the target tissue by an appropriate delivery device, such as a syringe, for example a Hamilton syringe.

According to the present invention, there is developed a system for the *Agrobacterium* mediated transformation of plants, preferably cereals, involving infection of target tissue. The system has been shown to be highly efficient, and very reproducible.

The target tissue may be any tissue which can subsequently be placed in a tissue culture phase and a plant regenerated. Particularly suitable target tissue, according to the present invention include an embryo, an inflorescence, an ovary, a leaf base, or an anther. The embryo, inflorescence, ovary or anther are preferably immature.

In another preferred embodiment of the invention, when the target tissue is an embryo, the target area for inoculation is the interface between two layers of cells that are in tight contact, i.e. the developing scutellum surface and adjoining starch parenchyma of endosperm. *Agrobacterium* has to be delivered to this interface with minimum damage to the target tissue to the extent that its regenerative capacity is not adversely affected. It could not be predicted from what is known in the field that such an effective, and reproducible, technique could be generated.

In the transformation method of the invention, the target tissue is inoculated and co-cultivated with *Agrobacterium.* Following this, a transgenic plant is regenerated by dedifferentiation and regeneration of the target tissue. Thus, following the inoculation and co-cultivation, the target tissue is made to dedifferentiate. From this dedifferentiated tissue a plant is obtained by standard procedures known in the art. Following inoculation and co-cultivation, the target tissue is preferably transferred into a more suitable environment for the required dedifferentiation and subsequent regeneration of a plant. Thus, at least part of the dedifferentiation of the target tissue (following inoculation and cultivation) is carried out *in vitro.* Regeneration of the plant is also preferably carried out *in vitro.*

One surprising feature of the method according to the present invention (at least for wheat immature embryos) is the frequent production of multiple transformation events from one isolated explant. In the art (Cheng et al, 1997) all plants derived from the same explant are usually considered to be clones of a given event. With this method, this assumption cannot be made as one explant frequently gives rise to several plants, each with a distinct integration pattern when analyzed by Southern blot. One possible explanation of this, which should not be interpreted as limiting to the invention, could be the absence of wounding of the most regenerable cells before the *Agrobacterium* is applied. More of the T-DNA transfers are likely to take place in cells that still have the capability to develop further.

One feature of cereal transformation, often described as crucial, is the induction of *Agrobacterium* with the inclusion, in the inoculation and/or co-cultivation media, of an *Agrobacterium vir* inducing agent (Hiei *et al.*, 1997, Cheng *et al.*, 1997). Such inducing agents include acetosyringone, vanillin, ferulic acid, catechol, and syringic acid. The present invention demonstrates successful *Agrobacterium* transformation in cereals where no inducing agent was necessary. In particular, successful *Agrobacterium* transformation of wheat was obtained with no inducing agent, showing that no inducing agent was necessary for efficient T-DNA delivery. Where the target tissue of the present invention is an immature embryo and its natural plant environment is provided by an immature seed, it is postulated that the *Agrobacterium* appears to be sufficiently induced naturally, by cells of the immature embryo. One possible explanation of this, which should not be interpreted as limiting to the invention could be that it is the cells which form the «natural plant environment » adjacent or around the target tissue which are responsible for the *Agrobacterium* induction. Removal of the embryo from its natural plant environment appears to deprive the target tissue of available substances which may assist in the *Agrobacterium* induction.

The present invention enables the introduction of a desired transgene or heterologous nucleic acid into plant tissue and the ability to obtain a fertile transgenic plant. It is particularly useful for the production of transgenic monocotyledonous plants since known transformation methods are associated with difficulties and low efficiencies of transformation. Suitable monocotyledonous plants include asparagus,

onion, oil palm, yam, banana, in particular any species from the Gramineae family, especially cereals (those grasses whose fruit are used for human food) such as wheat, barley, maize, rice, oats, rye, sorghum and millet.

This method is also applicable to dicotyledonous species, particularly where a tissue culture system exists, or may be developed, that includes a callus phase. Suitable dicotyledonous plants include rape, pea, pepper, soybean, sunflower, sugar beet and cucurbit and trees, such as rubber, pines and eucalyptus.

In accordance with the present invention, the heterologous nucleic acid is one which is not normally found in *Agrobacterium* T-DNA or the plant that is to be transformed. As used herein, the term heterologous nucleic acid includes all synthetically engineered and biologically derived genes which may be introduced into a plant by genetic engineering, including but not limited to non-plant genes, modified genes, synthetic genes, portion of genes, and genes from any plant species. The heterologous nucleic acid preferably contains the coding region of a protein or polypeptide or antisense molecule of interest, with flanking regulatory sequences that promote the expression thereof in the resulting monocot.

Methods for constructing heterologous nucleic acids for successful transformations of plants are well known to those skilled in the art, and the same methods of construction may be utilized to produce the heterologous nucleic acids useful herein. Weising et al. (1988) (Annual Rev. Genet. 22:241), the subject matter of which is incorporated herein by reference, describe suitable components which include promoters, polyadenylation sequences, selectable marker genes, reporter genes, enhancers, introns, and the like, and provide suitable references for compositions thereof. Sambrook et al. (1989) (Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, NY), provide suitable methods of construction.

Generally the plasmid comprising the nucleic acid heterologous gene will be relatively small, i.e. less than about 30kb, to minimize any susceptibility to physical, chemical, or enzymatic degradation which is known to increase as the size of the gene increases.

Suitable transgene or heterologous nucleic acids for use herein include all nucleic acids that will provide or enhance a beneficial feature of the resultant transgenic

plant. For example, the nucleic acid may encode proteins or antisense RNA transcripts in order to promote increased food values, higher yields, pest resistance, disease resistance, and the like. Representative nucleic acids include, for example, a bacterial dap A gene for increased lysine; Bt-endotoxin gene or protease inhibitor for insect resistance; lytic peptides genes for disease resistance, bacterial or plant EPSPS for resistance to glyphosate herbicide (US 4,940,835, US 5,188,642, US 4,971,908, US 5,145,783, US 5,312,910, US 5,633,435, US 5,627,061, US 5,310,667, WO 97/04103); bacterial or plant HPPD (WO 96/38567, WO 98/02562) for resistance to HPPD-inhibitor herbicides (i.e. diketones, isoxazoles, etc.), bar or pat genes for resistance to glufosinate, chitinase or glucan endo 1,3-B-glucosidase for fungicidal properties. Also, the nucleic acid may be introduced to act as a genetic tool to generate mutants and/or assist in the identification, genetic tagging, or isolation of segments of plant genes.

Examples of genes useful for modifying quality include: genes for starch biosynthetic or degrading enzymes e.g. starch synthases, starch branching enzymes (for example SBEI, SBEII, SSSI and DBEI from wheat disclosed in WO99/14314), and grain storage protein genes e.g. sub-unit proteins of glutenin (for example see WO97/25419), gliadins, hordeins. Artificial male sterility genes e.g. barnase (EP-A-0344029), and PR-glucanase (WO92/11379) under the control of a suitable promoter are also useful for the production of hybrid seed.

Genes may also be introduced for the purpose of producing pharmaceutically active compounds in plant or for improving the nutritional quality of plants (biopharming and functional foods).

Additional examples may be found in Weising, *supra*.

The plasmid comprising the heterologous nucleic acid to be introduced into the plant further will generally contain either a selectable marker or a reporter gene or both to facilitate identification and selection of transformed cells. Alternatively, the selectable marker may be carried on a separate vector and used in a cotransformation procedure. Both selectable markers and reporter genes may be flanked with appropriate regulatory sequences to enable expression in plants. Useful selectable markers are well known in the art and include, for example, antibiotic and herbicide resistance genes. Specific examples of such genes are disclosed in Weising et al, *supra*. A preferred

selectable marker gene is the sul gene conferring resistance to sulfonamides (EP-B-0369637). Other selectable markers known in the art include the hygromycin B phosphotransferase (*hpt*) coding sequence which may be derived from *E. coli*, the aminoglycoside phosphotransferase gene of transposon Tn5 (AphII) which encodes resistance to the antibiotics kanamycin, neomycin, and G418, as well as those genes which code for resistance or tolerance to glyphosate, bialaphos, methotrexate, imidazolinones, sulfonylureas, bromoxynil, dalapon, and the like. Selectable marker genes that confer herbicide tolerance are also of commercial utility in the resulting transformed plants.

Reporter genes which encode easily assayable marker proteins are well known in the art. In general, a reporter gene is a gene which is not present or expressed by the recipient organism or tissue and which encodes a protein whose expression is manifested by some easily detectable property, e.g. phenotypic change or enzymatic activity. Examples of such genes are provided in Weising et al, *supra*. Preferred genes include the chloramphenicol acetyl transferase (*cat*) gene from Tn9 of *E. coli*, the beta-gluronidase (*gus*) gene of the *uid*A locus of *E. coli*, the green fluorescence protein (GFP) gene from *Aequoria victoria*, and the luciferase (*luc*) gene from the firefly *Photinus pyralis*.

The regulatory sequences useful herein include any constitutive, inducible, tissue or organ specific, or developmental stage specific promoter which can be expressed in the particular plant cell. Suitable such promoters are disclosed in Weising et al, *supra*. The following is a partial representative list of promoters suitable for use herein: regulatory sequences from the T-DNA of *A. tumefaciens*, including mannopine synthase, nopaline synthase, and octopine synthase; alcohol dehydrogenase promoter from corn; light inducible promoters such as ribulose-biphosphate-carboxylase small subunit gene from a variety of species and the major chlorophyll a/b binding protein gene promoter; histone promoters (EP 507 698), actin promoters; maize ubiquitin 1 promoter (Christensen et al. (1996) Transgenic Res. 5:213); 35S and 19S promoters of cauliflower mosaic virus; developmentally regulated promoters such as the waxy, zein, or bronze promoters from maize; as well as synthetic or other natural promoters which are either inducible or constitutive, including those promoters exhibiting organ specific

expression or expression at specific development stage(s) of the plant, like the alpha-tubulin promoter disclosed in US 5,635,618.

Other elements such as introns, enhancers, polyadenylation sequences and the like, may also be present in the nucleic acid. These elements must be compatible with the remainder of the gene constructions. Such elements may or may not be necessary for the function of the gene, although they may provide a better expression or functioning of the gene by effecting transcription, stability of the mRNA, or the like. Such elements may be included in the nucleic acid as desired to obtain the optimal performance of the transforming gene in the plant. For example, the maize AdhlS first intron maybe placed between the promoter and the coding sequence of a particular heterologous nucleic acid. This intron, when included in a gene construction, is known to generally increase expression in maize cells of a protein. (Callis et al. (1987) Genes Dev. 1:1183). Other suitable introns include the first intron of the *shrunken-1* gene of maize (Maas et al. (1991) Plant Mol. Biol. 16:199); the first intron of the castor bean catalase (cat-1) gene (Ohta et al. (1990) Plant Cell Physiol. 31:805); potato catalase second intron of the ST-LSI gene (Vancanneyt et al. (1990) Mol. Gen. Genet. 220:245); tobacco yellow dwarf virus DSV intron (Morris et al. (1992) Virology 187:633; actin-1 (*act*-1) intron from rice (McElroy et al. (1990) Plant Cell 2:163); and triose phosphate isomerase (TPI) intron 1 (Snowden et al. (1996) Plant Mol. Biol. 31:689). However, sufficient expression for a selectable marker to perform satisfactorily can often by obtained without an intron. (Battraw et al. (1990) Plant Mol. Biol. 15:527).

The plasmid comprising the heterologous nucleic acid may also comprise sequences coding for a transit peptide, to drive the protein coded by the heterologous gene into the chloroplasts of the plant cells. Such transit peptides are well known to those of ordinary skill in the art, and may include single transit peptides, as well as multiple transit peptides obtained by the combination of sequences coding for at least two transit peptides. One preferred transit peptide is the Optimized Transit Peptide disclosed in US 5,635,618, comprising in the direction of transcription a first DNA sequence encoding a first chloroplast transit peptide, a second DNA sequence encoding an N-terminal domain of a mature protein naturally driven into the chloroplasts, and a third DNA sequence encoding a second chloroplast transit peptide.

To determine whether a particular combination of heterologous nucleic acid and recipient plant cells are suitable for use herein, the plasmid may include a reporter gene. An assay for expression of the reporter gene may then be performed at a suitable time after the heterologous nucleic acid has been introduced into the recipient cells. A preferred such assay entails the use of the *E. coli* beta-glucuronidase (*gus*) gene described by Jefferson et al. (1987) EMBO J. 6:3901, incorporated herein by reference.

A use of the present invention is the production of a fertile transgenic plant, which comprises one or more transgenes of interest. The seeds, or other propagating material from such a plant may be used to prepare subsequent generations of transgenic plants (including offspring) which comprise the one or more transgenes from the original method. Such subsequent generations of plants (including offspring), and propagating material, including seeds are also included in the scope of the present invention.

A second aspect of the invention provides the use of *Agrobacterium* in a transformation method comprising inoculation and co-cultivation of a target tissue with *Agrobacterium,* at a time when the target tissue is in its natural plant environment, followed by generation of dedifferentiated tissue from the target tissue.

The dedifferentiated tissue may optionally be regenerated into a transgenic plant. However, the second aspect of the invention is also advantageous in situations where the dedifferentiated tissue (itself, or any non-whole plant generated from it) is of use. Such situations include: storing of the dedifferentiated tissue for periods before further use; and recovery of useful plant products, such as secondary plant metabolites, for example, from cell culture. All preferred features of the first aspect of the invention, as described above, also apply to the second.

According to the first and second aspects of the invention, the transformed dedifferentiated tissue obtained may be regenerated. It may be regenerated to form, for example, callus tissue, whole plants, fertile whole plants, roots, shoots, seeds or other propagating material.

A third aspect of the invention provides the use of *Agrobacterium* in a transformation method comprising inoculation and co-cultivation of a target tissue with *Agrobacterium,* at a time when the target tissue is in its natural plant environment,

followed by generation of transgenic plant material via dedifferentiation and optionally regeneration of the target tissue.

The transgenic plant material obtained according to the third aspect of the invention may be callus, a whole plant (preferably fertile), roots or shoots, seeds or other propagating material.

All preferred features of aspects 1 and 2 also apply to the third aspect.

A fourth aspect of the invention provides transformed plant tissue obtained by a method according to the first or second aspects of the invention. Such transformed plant tissue includes callus, root material, shoot material, whole plants, seeds or other propagating material. The plants are most preferably fertile plants.

There are various reasons why the present invention is successful and why the target tissue is more susceptible to transformation by *Agrobacterium* while still in a natural plant environment. While not intending to limit the invention in any way, the following are proposed reasons as to why the present invention is successful:

1. The target cells, in their natural plant environment are rapidly dividing, probably more so than in tissue culture.
2. Avoiding a post-isolation treatment (i.e. inoculation and co-cultivation) increases the potential for callus formation and also regeneration potential
3. Different cells of the developing target tissue are exposed to *Agrobacterium* (compared to the art), specifically those that may be sub-epidermal and thought to be more regenerable
4. The absence of wounding (a pre-requisite for most other cereal transformation protocols) renders almost all cells that have been transformed capable of subsequent development.
5. Amalgamating the two steps of inoculation and co-cultivation reduces the stresses usually placed on the target tissue by these two separate tissue culture steps.
6. The natural environment of the seed is more propitious for normal cell development, in the presence of the *Agrobacterium*, than removal to a tissue culture environment.
7. Surface cells will be softer in any target tissue and provide less of a barrier to the *Agrobacterium* than once exposed to air.

The transformation method of the present invention can be described according to the following «general» methodology. A more detailed method is set out in the examples.

The following general methodology is described as applied to embryo inoculation (in the seed). The person skilled in the art will appreciate that the general method may be adapted to other target tissues.

Construct preparation and transfer to *Agrobacterium*

Binary, superbinary, pGreen or co-integrate vectors containing appropriate genes and selectable markers and/or reporter genes are transferred into *Agrobacterium* by one of various available methods e.g. triparental matings, electroporation. The *Agrobacterium* used can be any standard, usually disarmed, *Agrobacterium tumefaciens* or *rhizogenes* strain including, but not limited to,

LBA4404 (Hoekma *et al*, Nature (1983) 303:179-180)

EHA101 (Hood *et al*, J Bacteriol. (1986) 168:1291-1301

Disarmed C58, for example pMP90 (Koncz and Schell, M.G.G. (1986) 204, 383-396

LBA4404 containing pTOK233 (Hiei *et al*, Plant J (1994) 6:271-282)

Preparation of *Agrobacterium* for experiments

*Agrobacterium* is incubated in or on media with appropriate selective antibiotics at 25-30°C for 2 or 3 days. Bacteria is then collected and re-suspended in TSIM1 (MS media with 100mg/l myo-inositol, 10g/l glucose, 50mg/l MES buffer pH5.5) or another similar culture media, that may also containing acetosyringone. A wetter, e.g. pluronic acid F68 may also be included and other inducing agents for the *Agrobacterium* can optionally be used e.g. opines or other secondary plant metabolites.

Preparation of plant material

The starting material for this protocol is the inflorescence of a monocotyledonous (usually gramineous) plant, some time after anthesis has occurred. All stages of the inoculation and co-cultivation can be carried out on the inflorescence while it is still on the intact plant. However, for ease and containment purposes, removal of the parts of the plant that carry the inflorescence is preferred. Nevertheless, the inflorescence remains in its natural plant environment even when the plant part carrying it is removed from the plant.

For example, wheat tillers, or those from any other cereal, approximately 8-16 days post-anthesis are harvested from glasshouse or Conviron (controlled environment room) grown plants. Immature seed are then exposed, but left attached to the plant, by whatever means necessary. For example, in wheat, the glumes of each spikelet and the lemma from the first two florets are carefully removed to expose the immature seed. Only these two seed in each spikelet are generally uncovered. This procedure is carried out along the entire length of the inflorescence.

Inoculation of immature seed

*Agrobacterium* suspension is inoculated into the immature seed approximately at the position of the scutellum : endosperm interface, using any appropriate delivery device for example, a Hamilton syringe. The volume of bacteria suspension delivered is usually 1µl, but can vary depending on, for example, the seed size.

Tillers, for example, are then placed in water, or a nutritive solution, (optionally covered with a plastic bag to prevent seed dehydration) and placed in a lit incubator for 2-5 days (preferably 2 or 3 days). The temperature of the incubator can vary depending on the cereal species but will usually be in the range of 20-25°C.

Embryo isolation and culture

Following inoculation, immature seed are removed and surface sterilized. Immature embryos are isolated and placed on suitable callusing medium as exemplified by Weeks *et al*, Plant Physiol., 102:1077-1084, 1993; Vasil *et al.*, Biotech. 11: 1553-1558, 1993; Ishida *et al.*, 1996. Embryos are then successively transferred through any appropriate tissue culture procedure, including a selection step if required, that results in the regeneration of a transgenic plant, preferably a transgenic plant

The present invention will now be described with reference to the following, non-limiting examples.

In the examples the following figures are referred to:

Figure 1, which shows a cloning strategy for pSCVsulugi

Figure 2, which shows a plasmid map of pSCVsulugi.

Figure 3, which shows transient GUS expression in an immature embryo, histochemically stained 4 days after *in vivo* inoculation and co-cultivation, showing blue spots and « dashes ».

Figure 4, which shows areas of GUS expressing callus, histochemically stained one month after *in vivo* inoculation and co-cultivation, showing large dark blue stained areas.

Figure 5, which shows detail of figure 4: showing a dark blue-stained and highly delineated area of callus with potential for regeneration.

Figure 6, which shows a plasmid map of pSB11Sulugi.

Figure 7, which shows a plasmid map of pSCV1.2GI.

Figure 8, which shows transient GUS expression in soybean immature cotyledons.

**Example 1 Transformation of wheat by seed inoculation method - transient expression and production of transformed callus**

Construct preparation

For transformation purpose, the following constructs have been made (figure 1): The 4175bp HindIII fragment from pAHC25 (Christensen *et al*, Plant Mol. Biol.(1992) 18:675-689) was introduced in pIC19H (Marsh et al, Gene (1984) 32:481-485) cut with HindIII (resulting plasmid pAAA). The BamHI, SstI GUS-intron fragment from pUC-Top10-GUS INT (Weinmann *et al*, Plant J. (1994) 5:559-569) replaces the BamHI, SstI GUS fragment from pAAA to give pBBB. The XhoI, XbaI fragment containing the $Sul^R$ from pWP258 (described in patent application WO98/49316) is introduced in SalI, XbaI cut pSCV1 (Firek *et al*, Plant Mol. Biol.(1993) 22:129-142) creating pEEE. The HindIII pUbi-GUSint fragment from pBBB is cloned into pEEE cut by HindIII, to form pSCVSulugi (see Figure 2).

This construct was introduced into the disarmed supervirulent *Agrobacterium tumefaciens* strain EHA101, containing pEHA101 (Hood *et al*, J Bacteriol. (1986) 168:1291-1301) by electroporation and subsequent selection on 50mg/l kanamycin and 70mg/l gentamycin.

Preparation of *Agrobacterium* for experiments

*Agrobacterium* was incubated on solidified YEP media with 20mg/l kanamycin sulphate at 27°C for 2 days. Bacteria was then collected and re-suspended in TSIM1 (MS media with 100mg/l myo-inositol, 10g/l glucose, 50mg/l MES buffer pH5.5) containing 400µM acetosyringone to an optical density of 2.4 at 650nm.

Preparation of plant material

Wheat tillers of NB1 (a Spring wheat variety obtained from Nickerson Seeds Ltd, Rothwell, Lincs.), approximately 14 days post-anthesis (embryos approximately 1mm in length) were harvested from glasshouse grown plants to include 50cm tiller stem, (22/15°C day/night temperature, with supplemented light to give a 16 hour day). All leaves were then removed except the flag leaf and the flag leaf cleaned to remove contaminating fungal spores. The glumes of each spikelet and the lemma from the first two florets were then carefully removed to expose the immature seed. Only these two seed in each spikelet were generally uncovered. This procedure was carried out along the entire length of the inflorescence. The ears were then sprayed with 70% IMS as a brief surface sterilization.

Inoculation of tillers

*Agrobacterium* suspension (1µl) was inoculated into the immature seed approximately at the position of the scutellum : endosperm interface, using a 10µl Hamilton syringe, so that all exposed seed were inoculated. Tillers were then placed in water, covered with a translucent plastic bag to prevent seed dehydration, and placed in a lit incubator for 3 days at 23°C, 16hr day, 45µEm$^{-2}$s$^{-1}$ PAR.

Embryo isolation and culture

After 3 days of co-cultivation, inoculated immature seed were removed and surface sterilized (30 seconds in 70% ethanol, then 20 minutes in 20% Domestos, followed by thorough washing in sterile distilled water). Immature embryos (136 in total) were aseptically isolated and placed on W3 media (as described in patent application WO98/49316) with the addition of 150mg/l Timentin (W3T) and with the scutellum uppermost (20 embryos per plate). Cultures were placed at 25°C in the light (16 hour day, 80µEm$^{-2}$s$^{-1}$ PAR).

After 3 days culture on W3T, 50 embryos were removed and put in X-gluc solution (Jefferson, Plant Mol. Biol. Rep.(1987) 5:386-405) at 37°C for 16 hours, to assess GUS expression. The development of the embryonic axis on the remaining embryos was assessed 5 days after isolation and the axis was removed where necessary to improve callus production. Eight days post isolation a further 31 embryos were removed and stained as before.

The remaining 55 embryos were maintained on W3T for 4 weeks, with a transfer to fresh media at 2 weeks post-isolation.

One month after embryos were isolated, remaining embryo-derived callus was assessed for embryogenic capacity and stained for GUS expression.

Results

Histochemical Staining 4 days post-inoculation

Some isolated embryos showed evidence of needle damage as a result of the inoculation procedure. This was very rarely associated with any of the GUS expression determined histochemically.

GUS expression in these embryos appeared in three forms

1. Standard blue GUS spots as documented in the art, see Fig 3
2. Small dashes of blue comprising several linked cells all apparently expressing GUS to the same extent, see Figure 3
3. Large blocks of dark blue staining on the scutellum and the embryonic axis that started as spots or dashes and rapidly invaded large areas of tissue so that quantification was impossible.

The combination of scores from 1. and 2. gave an average of 6 spots per embryo with a range of 0-64 spots.

Control embryos (30) derived from inoculations with the EHA101 carrying only pEHA 101 and no vector plasmid strain produced no blue staining of any sort with X-gluc. No staining of EHA101 containing SCVsulugi was observed either.

Histochemical staining 14 days post-inoculation

The staining pattern in these embryos was slightly different to that seen at 4 days. The staining was usually in the form of small spots, or sometimes as small zones. The average number of spots/zones per embryo was 3, with a range of 0-25. The embryo with the maximum number of staining events, also had more of the less commonly observed blue 'zones' on the scutellar tissue.

Callus development

After 4 weeks growth, callus derived from the inoculated embryos was very similar to control callus obtained from uninoculated embryos. Presence of the bacteria

did not appear to have substantially reduced the embryogenic capacity of the callus derived from the inoculated embryos.

Histochemical staining one month post-inoculation

Of the remaining 55 immature embryo-derived calli that were stained in x-gluc 16 showed evidence of GUS expression in the form of darkly stained blue cells. In 6 of these calli, quite large dark blue regions of staining were observed, up to 1mm in diameter, and appearing as highly delineated areas, see Figure 4. Three of the blue regions showed three-dimensional structure in the form of cell protrusions from the callus surface (as in Figure 5), and were assessed as being in embryogenic callus, with good potential for regeneration.

The recovery of three stable integration events with good regeneration potential from this experiment, suggests that this method has a high transformation efficiency.

**Example 2 Transformation of wheat using seed inoculation method - transformation and regeneration of transgenic plants**

As for example 1 except that 187 embryos were inoculated and isolated, and these were subjected to a selection step.

Selection of transformed callus

After 12 days cultivation on W3T, embryogenic calli were transferred to W3 media with 2mg/l Asulam and 150mg/l Timentin (W32AT). Calli were maintained on this media for a further 2 weeks and then each callus was divided into 2mm pieces and re-plated onto W32AT.

After a further 2 weeks culture, all tissue was assessed for development of embryogenic callus: any callus showing signs of continued development after 4 weeks on selection was transferred to regeneration media (RMT - MS with 40g/l maltose and 150mg/l Timentin, pH5.8, solidified with 6g/l agarose, Sigma type I). Shoots were regenerated within 4 weeks on this media and then transferred to MS30 with 150mg/l Timentin for shoot elongation and rooting.

Results

Transformation was determined by one or more of the following methods :

a) GUS histochemical staining (Jefferson, 1987) on at least roots and leaves

b) PCR analysis for the sul gene.

PCR analysis was performed on genomic DNA extracted from 1-2cm$^2$ fresh leaf material using miniprep method described by Stacey and Isaac (Methods in Molecular Biology, Vol.28: Protocols for nucleic acid analysis by nonradioactive probes, 9-15, Humana Press Inc., Totawa, NJ (1994)). PCR reaction were performed using primers designed to amplify a 380bp Sul fragment (5' TTGTGCGGTTCTTCGAGGCG 3' and 5' TGCGCTTCGCAGATCTCCAG 3'. Reactions conditions were as followed "hot start" (94°C, 3 min) followed by 30 cycles of denaturation (95°C, 30s), annealing (60°C, 30s), extension (73°C, 2min) followed by 1 cycle at 73°C (5min) and then held at 24°C.

c) Southern analysis.

Southern analysis was performed on DNA from a full scale (9ml) extraction from lyophilized ground tissue (Stacey and Isaac, 1994).DNA samples were adjusted to 0.2mg/ml and digested with restriction enzymes HindIII, EcoRI and KpnI. Restriction enzyme digestion, gel electrophoresis and vacuum blotting were carried out as described by Stacey and Isaac (1994). Digoxygenin-labelled Sul and GUS probes were produced by PCR according to the method of McCreery and Helentjaris (Methods in Molecular Biology, Vol.28: Protocols for nucleic acid analysis by nonradioactive probes, 67-71, Humana Press Inc., Totawa, NJ (1994)). Hybridization of the probes to the Southern blot and detection by chemiluminescence was performed according to the method of McCreery and Helentjaris (Methods in Molecular Biology, Vol.28: Protocols for nucleic acid analysis by nonradioactive probes, 107-112, Humana Press Inc., Totawa, NJ (1994)).

d) Segregation analysis of the T1 generation.

Analysis was performed by histochemical staining on germinated seedlings.

2 Plants representing 2 separate transformation events (1.1% efficiency) were regenerated, leaf and root samples of which showed strong GUS expression by histochemical staining. Stable transformation was confirmed by Southern analysis and assessment of gene segregation in the progeny.

In a separate experiment, 116 embryos have been inoculated and 4 separate GUS posisite transgenic lines regenerated.

The efficiencies obtained (1.1 and 3.4 %) are comparable with those obtained with other combinations of vectors and bacterial strains (see example 5).

**Example 3 Transformation of maize by seed inoculation method - transient expression, production of transgenic callus and regeneration of transformed plants.**

Construct preparation

As for example 1.

Preparation of *Agrobacterium* for experiments

*Agrobacterium* is incubated on solidified YEP media with appropriate antibiotics at 27°C for 2 days. Bacteria is then collected and re-suspended in TSIM1 (MS media with 100mg/l myo-inositol, 10g/l glucose, 50mg/l MES buffer pH5.5) containing 100-400μM acetosyringone to a density of 2.0-2.4 at 650nm.

Preparation of plant material

Sections of maize plants, variety A188, (glasshouse grown at 20-35°C, 16 hr day) are excised to include at least the stem node below and the stem node above an ear/cob, 6-14 days post anthesis, and with at least one leaf retained. The husk leaves of the cob are carefully pulled down to expose the immature seed, and all silks removed.

With a sharp implement, every second longitudinal row of immature seed is carefully removed and discarded, and the whole lightly sprayed with 70% ethanol.

Inoculation of maize ears

Inoculation as for example 1. Plant sections are subsequently placed in water and the husk leaves replaced over the cob to prevent seed dehydration - covering with a plastic bag may also be advised. Material is then placed in a lit incubator at 23-25°C for 2-5 days.

Embryo isolation and culture

As described by Ishida *et al*, 1997. Embryos are removed 2 days post isolation for transient expression analysis, and the remainder subjected to a selection step in order to regenerate stably transformed maize plants.

**Example 4 Transient expression in immature wheat embryos following seed inoculation, in the absence of the inducer acetosyringone.**

Construct preparation

As for example 1.

Preparation of *Agrobacterium* for experiments

As for example 1 with the exception that acetosyringone was excluded from the inoculation media, and a lower concentration of *Agrobacterium* was used (OD 2.1 at 650nm).

Preparation of plant material

As for example 1.

Inoculation of tillers

As for example 1.

Embryo isolation and culture

Isolation as for example 1. After 2 days on W3T, 77 embryos were assessed for GUS expression by histochemical analysis in X-gluc.

Results

Blue spots/dashes were visible on the upper and lower surfaces of the scutellum, and in many cases, spots appeared to be incorporated into the scutellum structure, that is, were between the upper and lower epidermis. A few embryos had no evidence of GUS expression at all. The mean number of spots per embryo was 25.4 with a range of 0-252.

Other Embodiments

It is to be understood that, while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not to limit the scope of the invention. Other aspects, advantages and modifications are within the scope of the claims as set forth below.

**Example 5 Stable transformation of wheat by seed inoculation method.**

Preparation of Construct and introduction to *Agrobacterium* strain LBA4404

The XhoI, XbaI fragment containing the $Sul^R$ from pWP258 ( See example 1) is introduced in XhoI, XbaI cut pSB11 (Komari *et al.*, Plant J. (1996) 10:165-174) creating pFFFII. The HindIII pUbi-GUSint fragment from pBBB ( See example 1) is cloned into pFFFII cut by HindIII, to form pSB11Sulugi (see Figure 6).

This construct was introduced into *Agrobacterium tumefaciens* strain LBA4404(pSB1) (Komari *et al.*, 1996), by electroporation and subsequent selection on 50mg/l Spectinomycin to form super binary vector pSB111Sulugi by recombination.

Preparation of bacteria for inoculation.

*Agrobacterium* was grown and resuspended using the method from example 1 but with varying amounts of acetosyringone (0-400μM) in the inoculation media.

Inoculation of seed

Method for example 1, experiments containing 50-300 embryos, see Table 1.

Tissue culture of isolated embryos

See example 2.

Results

See Table 1.

Data in Table 1 represents successful experiments - the few experiments that did not yield plants were excluded. Transformation was determined by one or more of the following methods :

a) GUS histochemical staining (Jefferson, 1987) on at least roots and leaves

b) PCR analysis for the sul gene.

c) Southern analysis.

d) Segregation analysis of the T1 generation.

Transformation efficiencies

Transformation efficiencies of successful experiments ranged from 0.5-5.8%, with a mean of 1.5%. Transformed plants were regenerated from experiments initiated both with and without the inducer acetosyringone in the inoculation media.

Transmission of the GUS gene into the T1 generation was confirmed for several lines, see Table 1.

The transformation efficiencies obtained were comparable or higher than any wheat transformation efficiencies published (Vasil *et al.*, Bio/Technology (1992), 10: 667-674, Weeks *et al.*, Plant Physiol. (1993), 102: 1077-1084, Nehra *et al.*, Plant J. (1994), 5: 285-297, Becker *et al.*, Plant J. (1994), 5: 299-307, Zhou *et al.*, Plant Cell Rep. (1995), 15: 159-163, Cheng *et al.*, (1997))

Integration patterns

Gene integration patterns of transformed lines ranged from single insertions with Mendelian patterns of inheritance to multiple copy number lines with up to seven copies of the T-DNA.

**Example 6 Transformation of maize by seed inoculation method - transient expression and regeneration of transformed plants.**

Construct preparation

As for example 1.

Or LBA 4404 (pSB131) described by Ishida *et al.*, 1997.

Preparation of *Agrobacterium* for experiments

*Agrobacterium* was incubated on solidified YEP media with appropriate antibiotics at 27°C for 2 days. Bacteria was then collected and re-suspended in TSIM1 (MS media with 100mg/l myo-inositol, 10g/l glucose, 50mg/l MES buffer pH5.5) containing 100-400μM acetosyringone with 0-0.5% Pluronic acid F68 to a density of 2.0-2.4 at 650nm.

Preparation of plant material

Sections of maize (*Zea mays* L.) plants, variety A188 or Hi II, (glasshouse grown at 20-35°C, 16 hr day) were excised to include at least the stem node below and the stem node above a cob, 6-14 days post anthesis, and with at least one leaf retained. The husk leaves of the cob were carefully pulled down to expose the immature seed, and all silks removed. The cob was lightly sprayed with 70% ethanol for sterilization.

Inoculation of maize ears

Inoculation as for example 1. Plant sections were subsequently placed in water and the husk leaves replaced over the cob covered and with cling film to prevent seed dehydration. Material was then placed in a lit incubator at 22-25°C for 2-5 days.

Embryo isolation and culture

After co-cultivation the cob was sterilized 20 minutes in a 20% Domestos solution. The embryos were then aseptically isolated, rinsed twice in LSinf (Ishida *et al*, 1997) supplemented with 250mg/l Cefotaxime and transferred to callus induction medium LSD (Ishida *et al*, 1997) for 2-10 days in the dark at 25°C.

Embryos were removed 2 days post isolation for transient expression analysis, and the remainder subjected to a selection step in order to regenerate stably transformed maize plants as described by Ishida *et al*, 1997.

Results

As shown in table 2, inoculation of immature embryos within the seed for maize led to transfer of T-DNA and expression of GUS gene with either strain used and for both varieties. Although only 3-10% of the immature embryos expressed GUS after co-cultivation, phosphinothricin resistant plants have been regenerated and expressed the GUS gene. The transformation frequencies could be considered as relatively high considering that the numbers of embryos put through selection for stable transformation was low. It also indicates that even if the T-DNA transfer is lower than a traditional full *in vitro* system (Ishida *et al*, 1997), the inoculation of the embryo in its natural seed environment targets cells that have a better potential for regeneration.

These results also show that this method is applicable to other monocot species and is not variety dependent regarding to the transformation step.

**Example 7 Production of transgenic *Brassica napus* plants by inoculation of Agrobacterium into the base of cotyledonary petioles.**

Construct preparation

P35S-nptII-tNOS Hind III fragment isolated from pCaMVNEO (Fromm *et al.*, Nature (1996), 319: 791-793) was inserted into pSCV1 (Firek *et al*, Plant Mol. Biol. (1993) 22:129-142) to give pSCV1.2. The p35S-gus-intron-polyACaMVHind III fragment (Vancanneyt *et al.*, M.G.G. (1990), 220: 245-250) was inserted in the Sma I site of pSCV1.2, resulting in pSCV1.2GI (Figure 7)

This construct was introduced into *Agrobacterium tumefaciens* strain C58pMP90 (Koncz and Schell, 1986).

Seedling preparation

Seeds of *Brassica napus* RV31, a spring variety, were surface sterilized using 15% Domestos for 20 minutes, followed by extensive washing with sterile water, to remove fungal and bacterial pathogens. Seeds (110) were then placed on germination media (MS media with 20g/l sucrose) in Beatson jars (10 seed per jar) and placed at

25°C with a 16hr photoperiod for 3 days. Seedlings thus germinated are at the stage where the cotyledons and associated petioles have emerged but are not fully expanded.

Preparation of *Agrobacterium*

C58pMP90 SCV1.2GI was inoculated into 10ml of mg/l media with appropriate antibiotic selection and grown at 28°C on a rotary shaker for approximately 24 hours. The overnight culture was then centrifuged at 2000 rpm for 20 minutes and the supernatant discarded. Pelleted bacteria were re-suspended in MS30 liquid (MS media containing 30g/l sucrose) to an $OD_{650nm}$ of approximately 2.0 (2.175).

Inoculation of *Agrobacterium*

The bacterial suspension (0.5-1.0µl) was injected into the area at the base of each cotyledonary petiole using a 10µl Hamilton syringe. Seedlings were then transferred to 20°C for 2 days.

Callus induction and plant regeneration

Cotyledons were excised from the seedling and cultured essentially as per the method of Moloney *et al.*, Plant Cell Reports (1989) 8: 238-242. The surface of excised *Brassica napus* cotyledonary petioles cultured in this way undergo a brief period of callus development from the exposed vascular bundle tissue before shoot meristems form in this callus, within 8 days of culture (Ono *et al.*, Plant Cell Reports (1994) 14: 13-17).

Results

6 Transformed shoots were regenerated from the 200 excised cotyledonary petioles, as determined by x-gluc staining for the GUS gene and PCR analysis for the NptII gene, equivalent to a 3.0% transformation efficiency. 1 Further line was shown to contain the gene by PCR analysis but had no GUS activity by x-gluc staining. Analysis of the T1 generation of 5 of the GUS expressing transformed lines by x-gluc staining showed transmission of the GUS gene to the next generation with the following results:

T1 Plants assessed for GUS activity

| Line | Positive | Negative | Ratio |
|---|---|---|---|
| 1 | 10 | 10 | 1:1 |
| 2 | 9 | 0 | 9:0 |
| 3 | 21 | 0 | 21:0 |
| 4 | 19 | 0 | 19:0 |
| 5 | 14 | 1 | 14:1 |

Discussion

Inoculation of *Agrobacterium* into the base of cotyledonary petioles while they are still attached to the seedling represents a marked departure from the published transformation system where the petioles are excised first and then the *Agrobacterium* applied. Although physically difficult to perform, this method proved to be remarkably efficient with little practice. With several years experience, using the standard published method and the same *Brassica napus* variety, a routine 5-10% transformation efficiency can be obtained. To achieve 3.0% at the second attempt (an initial experiment achieved 1 transformed shoot from 80 explants - 1.25%) using this new method is surprising. This further demonstrates the applicability of this method of gene delivery to any species where a tissue culture system with a callus phase exists - monocotyledon or dicotyledon.

**Example 8 : Transformation of soybean by seed inoculation method - transient expression.**

Construct preparation

*Agrobacterium tumefaciens* strain LBA 4404 was transformed with the super-binary vector pVec 035 containing the GUS intron gene driven by the CaMV 35S promoter (Supplied by B. Pelissier, Aventis Crop Science, Lyon, Fr).

Preparation of *Agrobacterium* for experiments

*Agrobacterium* was incubated on solidified YEP media with appropriate antibiotics at 27°C for 2 days. Bacteria was then collected and re-suspended in TSIM1 (MS media with 100mg/l myo-inositol, 10g/l glucose, 50mg/l MES buffer pH5.5) containing 0-400µM acetosyringone to a density of 0.5-2.0 at 650nm.

Preparation of plant material

Soybean plants *Glycine max* cv Jack were grown in glasshouse at a temperature of 23-25°C, with supplemented light to give a 14 hour day.

Inoculation of soybean seeds

Immature seeds were inoculated when the embryos were 3-7 mm in size. The injection of 0.5-1µl of *Agrobacterium* suspension was performed as described in example 1 by delivering the suspension between the two cotyledons, through the pod and longitudinally to the embryo. The plants were then incubated at 23-25°C for 2-5 days.

Embryo isolation and culture

After co-cultivation the immature seeds were removed and sterilized 20 minutes in a 20% Domestos solution. The embryos were then aseptically isolated transferred to callus induction medium MSI (MS medium and B5 vitamins with 60 g/l sucrose and 40 mg/l 2,4-D, solidified with 3 g/l Phytagel, adjusted to pH 7) supplemented with 350mg/l Cefotaxime in light conditions at 27°C. After 2-10 days the embryos were used for histochemical GUS staining to assess T-DNA transfer efficiency.

Results

As shown in table 3, inoculation of soybean immature embryos within the seed and pod led to transfer of T-DNA and expression of GUS. GUS positives spots or areas were widely spread over the immature embryos and not necessarily associated with the wounding sites (Figure 8). Unlike the SAAT transformation of soybean cotyledon method (Santarèm *et al.*, Plant Cell Report (1998), 17: 752-759) this technique provides an easier transformation protocol and a higher regeneration potential as the target cells are not wounded.

**Example 9 : Transformation of sunflower by seed inoculation method - transient expression.**

Construct preparation

C58C1(pGV2260) (Simpson et al., Plant Mol. Biol. (1986), 6: 403-416) (pBin 19) (Bevan, Nuc. Acids Res. (1984), 12: 8711-8121)

C58pMP90 (pSCV1.2GI) (See example 7)

Preparation of *Agrobacterium* for experiments

*Agrobacterium* was incubated on solidified YEP media with appropriate antibiotics at 27°C for 2 days. Bacteria was then collected and re-suspended in TSIM1 (MS media with 100mg/l myo-inositol, 10g/l glucose, 50mg/l MES buffer pH5.5) containing 0-400µM acetosyringone to a density of 2.0-2.4 at 650nm.

Preparation of plant material

Sunflower plants *Helianthus annuus* cv HA300B were grown in glasshouse 15-30°C with supplemented light to give a 14 hour day.

Inoculation of sunflower seeds

Immature seeds were inoculated 10 to 25 days post-anthesis. The injection of 1µl of *Agrobacterium* suspension was performed as described in example 1 through the micropyle to be delivered between the two cotyledons. The capitulum was then incubated at 22-25°C for 2-5 days.

Embryo isolation and culture

After co-cultivation the immature seeds were removed and sterilized 20 minutes in a 20% Domestos solution. The embryos were then aseptically isolated, transferred to callus induction medium (MS with 30g/l sucrose, solidified Agar-agar 10g/l, pH 5.7 and supplemented with 0.5 mg/l NAA, 0.5 g/l BAP and 500 mg/l Cefotaxime) and cultured at 21-24°C, 16hr day, 30µEm$^{-2}$s$^{-1}$ PAR. After 2-10 days the embryos were used for histochemical GUS staining to assess T-DNA transfer efficiency.

Results

As shown in table 4, inoculation of sunflower immature embryos within the seed led to transfer of T-DNA and expression of GUS gene with either strain used (5.9%-65.4%). GUS positives spots were mainly located on the cotyledons, but transformation events have been also located on the hypocotyl. Only two experiments have been laid

down to assess the potential of the seed inoculation method to transform sunflower immature embryos. Surprisingly it has proven to be very efficient even if a critical parameter seems to be the development of the immature embryo.

# CLAIMS

1. A transformation method comprising inoculation and co-cultivation of a target tissue, from a target plant, with *Agrobacterium,* at a time when the target tissue is in its natural plant environment, followed by generation of a transgenic plant via dedifferentiation and regeneration of the target tissue.

2. A transformation method, as claimed in claim 1, wherein the transgenic plant is a fertile plant.

3. A transformation method comprising inoculation and co-cultivation of a target tissue, from a target plant, with *Agrobacterium,* at a time when the target tissue is in its natural plant environment, followed by generation of dedifferentiated tissue from the target tissue.

4. A transformation method as claimed in one of claims 1 to 3, wherein wounding of the target cells in the target tissue is kept to a minimum or totally excluded.

5. A transformation method, as claimed in one of claims 1 to 4, wherein at least some of the dedifferentiation of the target tissue is carried out *in vitro.*

6. A transformation method, as claimed in any one of claims 1 to 5, wherein the target plant is a dicotyledonous or a monocotyledonous species.

7. A transformation method as claimed in claim 6, wherein the target plant is of the Gramineae family.

8. A transformation method, as claimed in claim 6, wherein the target plant is rape, pepper, soybean, sunflower, sugar beet or a cucurbit.

9. A transformation method, as claimed in anyone of claims 1 to 8, wherein the target tissue is an embryo, an inflorescence, an ovary, a leaf base, or an anther.

10. A transformation method, as claimed in any one of claims 1 to 7, wherein the target tissue is an immature embryo, an immature inflorescence, an immature ovary or an immature anther.

11. A transformation method as claimed in claim 10, wherein the target area for inoculation is the interface between two layers of cells that are in tight contact.

12. A transformation method, as claimed in any one of claims 1 to 11, wherein no *Agrobacterium vir* inducing agent is added around the time of the *Agrobacterium* inoculation.

13. A transformation method as claimed in any one of claims 1 to 12, wherein no *Agrobacterium vir* inducing agent is added around the time of the *Agrobacterium* co-cultivation.

14. Use of *Agrobacterium* in a transformation method comprising inoculation and co-cultivation of a target tissue, from a target plant, with *Agrobacterium,* at a time when the target tissue is in its natural plant environment, followed by generation of transgenic plant material via dedifferentiation of the target tissue.

15. Use of *Agrobacterium,* as claimed in claim 14, wherein the transgenic plant material is dedifferentiated and optionally regenerated to form callus, root, shoot or plant (preferably fertile) material.

16. Transformed plant tissue obtained by a method of any one of claims 1 to 13.

17. Transformed plant tissue, as claimed in claim 16 which is callus, root, shoot or whole plant (preferably fertile) material.

18. Transformed plant tissue obtained from plant tissue as claimed in claim 16 or claim 17.

19. Transformed plant tissue as claimed in claim 18, which is seed or other propagating material.

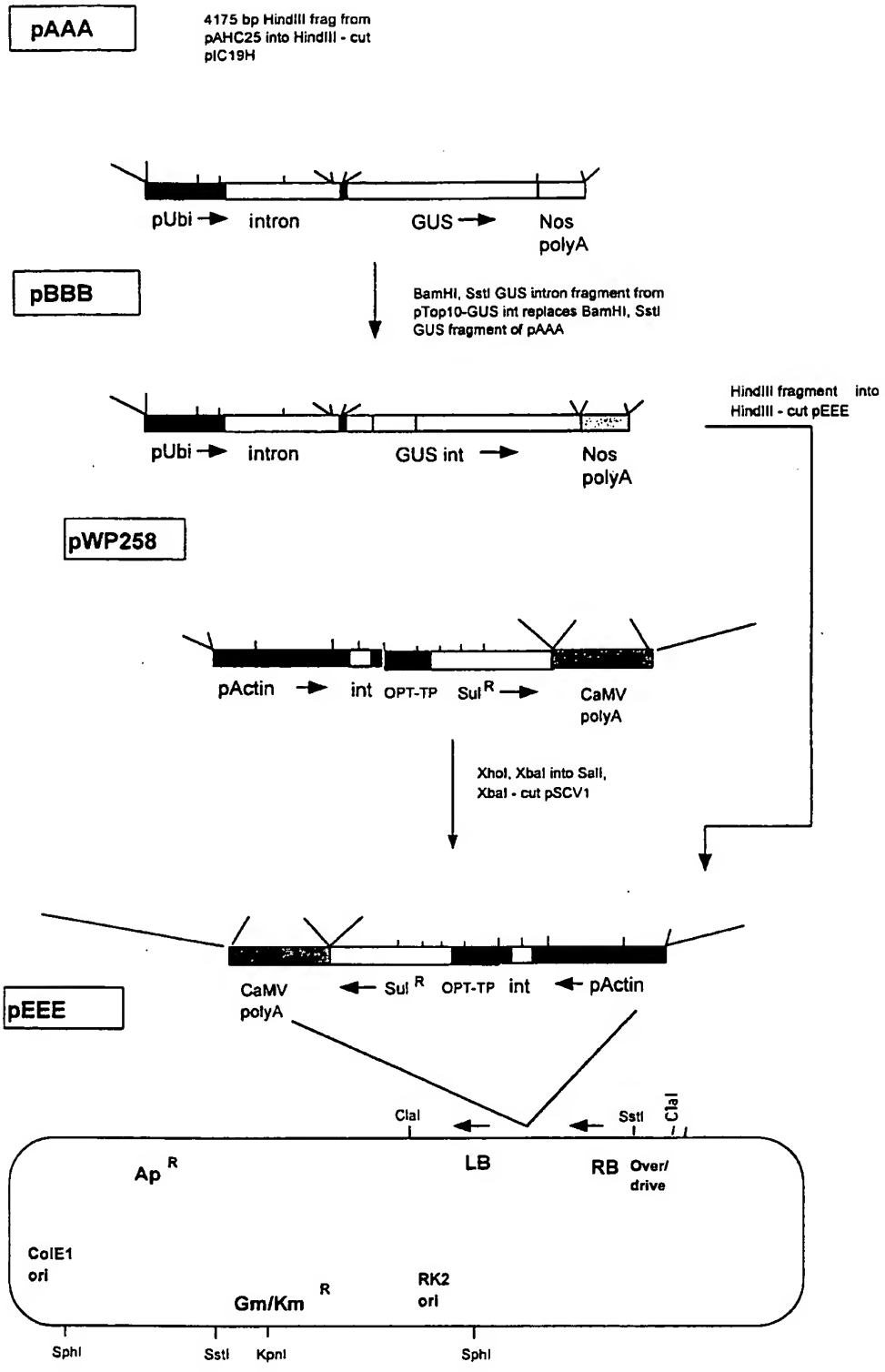


Figure 1: Cloning strategy for pSCV sulugi

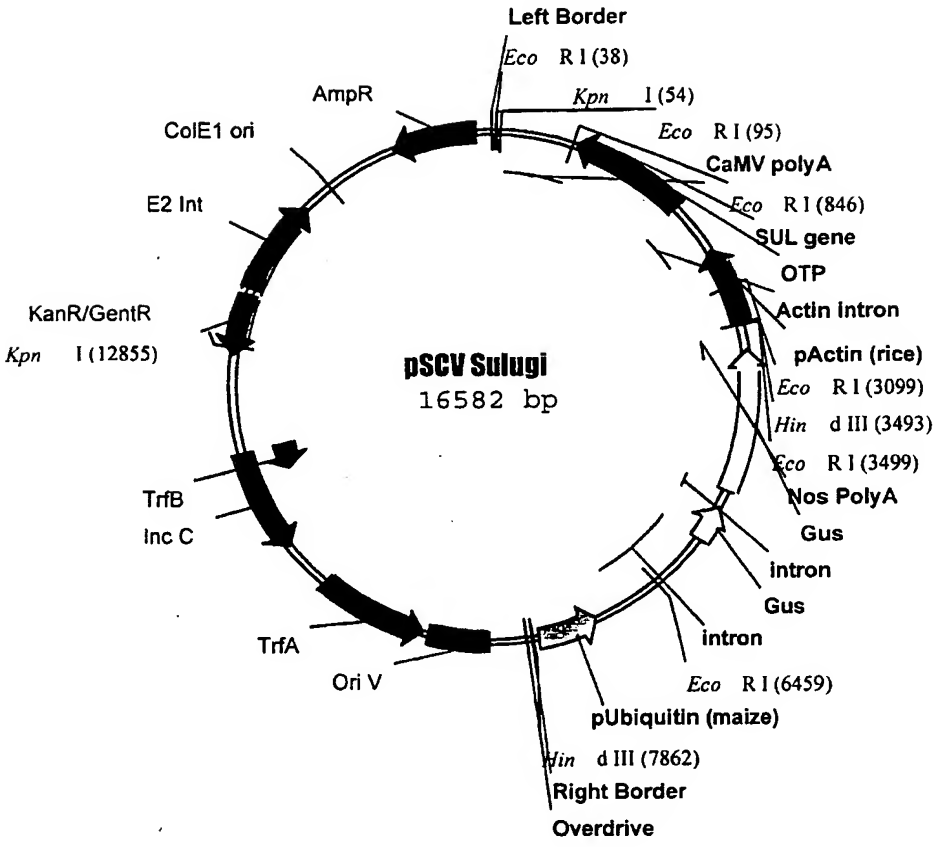


Figure 2 : pSCV Sulugi

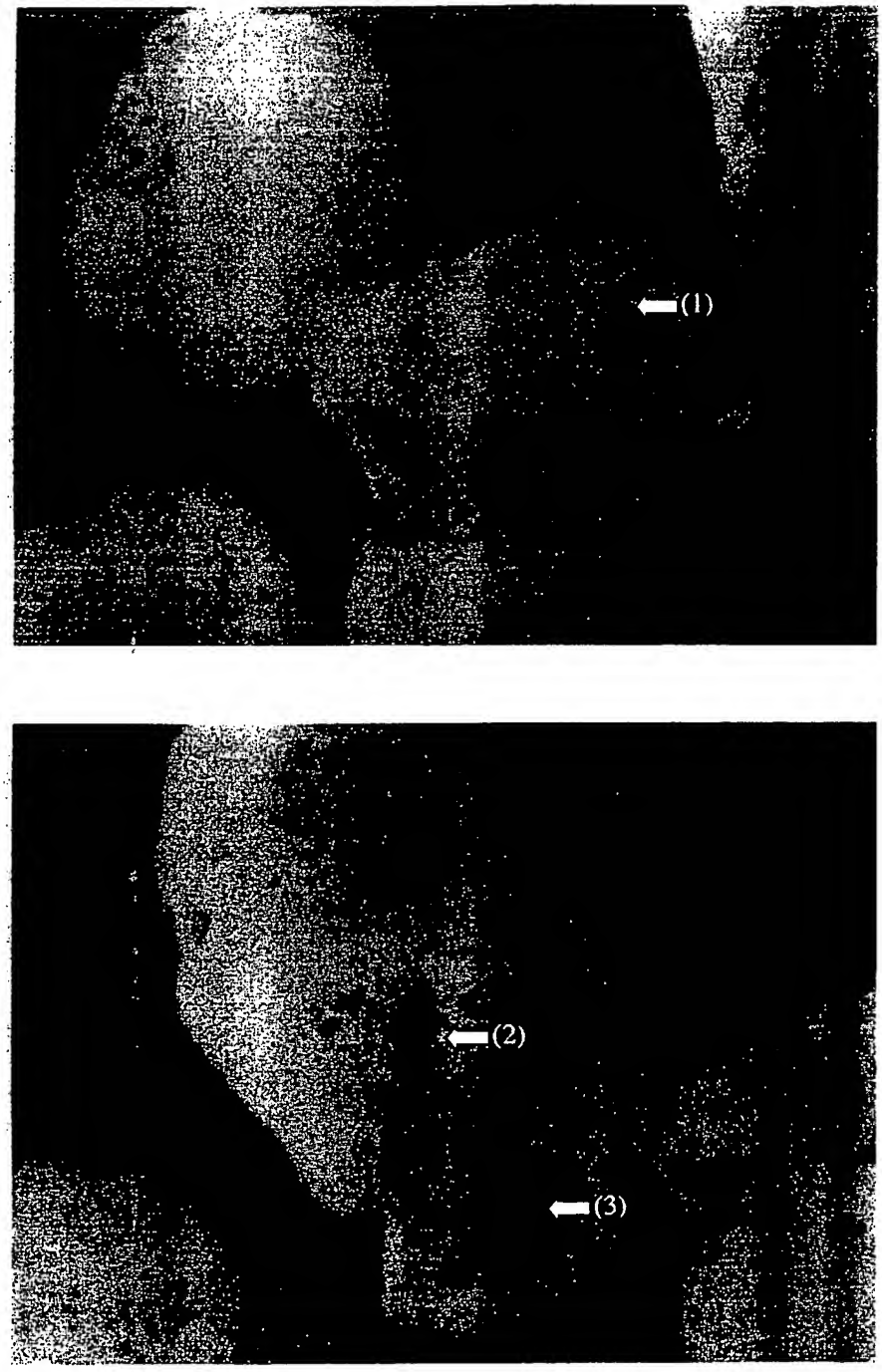


Figure 3 : Transient expression with wheat immature embryos
(1) Standard blue GUS spots
(2) Small blue dashes comprising several linked cells
(3) Large areas of blue staining

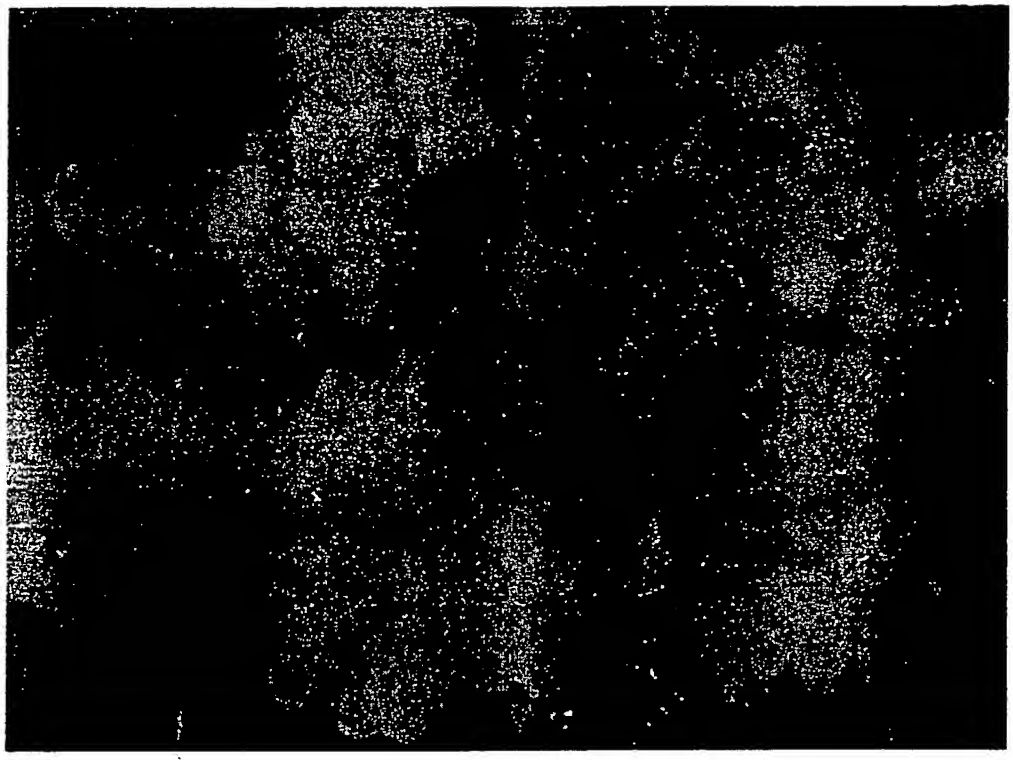

Figure 4 : Stable GUS expression in wheat callus

Figure 5 : Stable GUS expression (detail) in wheat callus

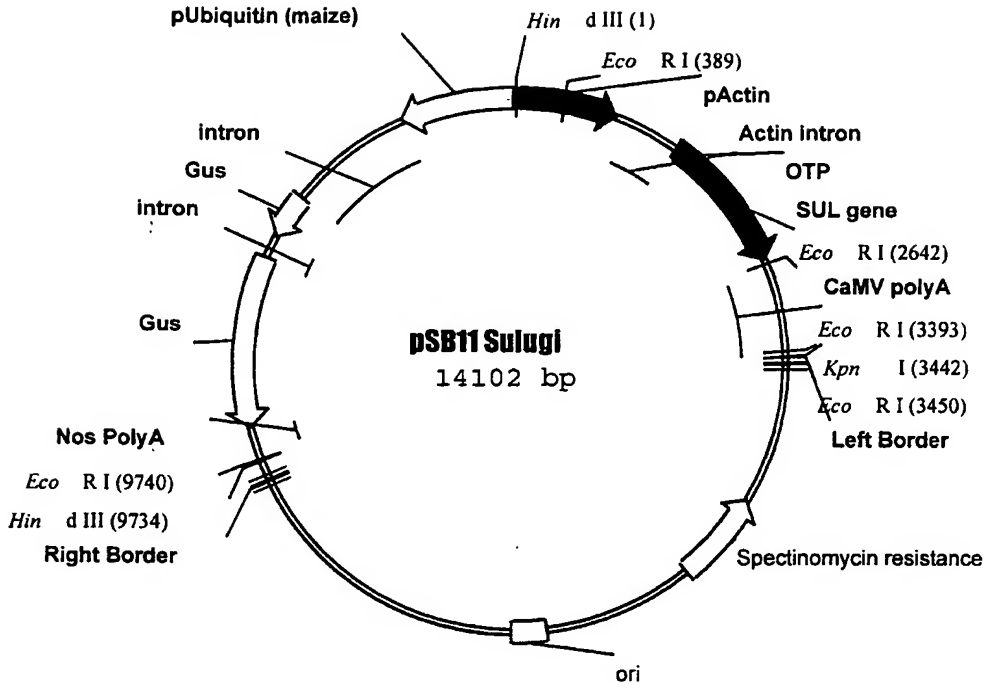


Figure 6: pSB11Sulugi

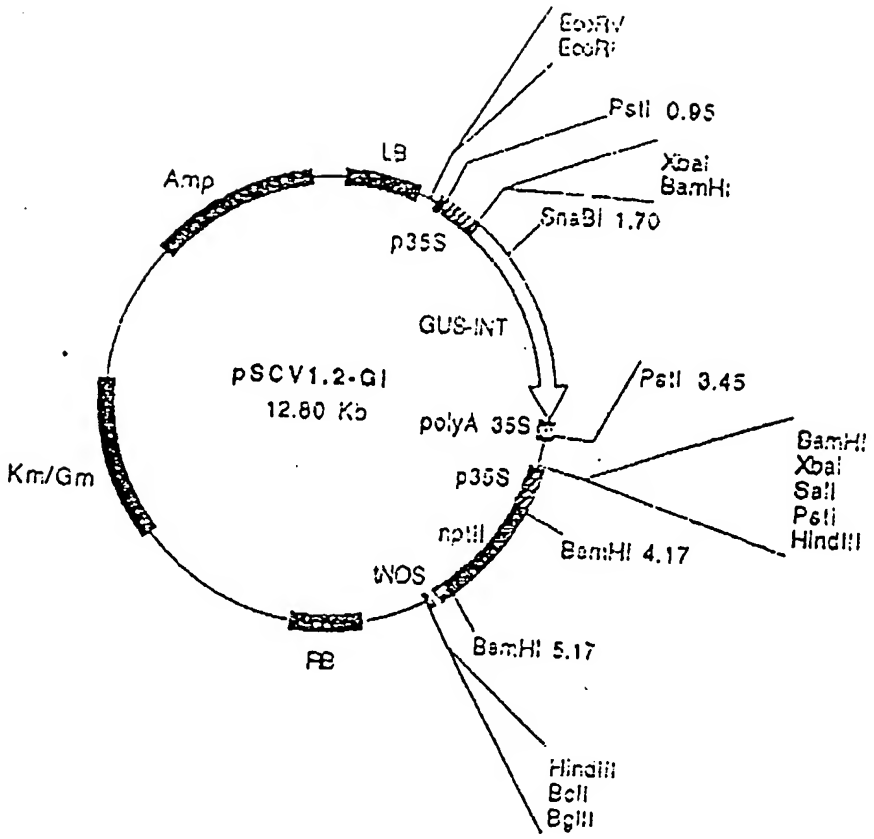


Figure 7 : pSCV1.2GI

Figure 8 : Example of transient GUS expression in soybean immature cotyledons

| Experiment | Treatment | Number Embryos Isolated (X) | Number of embryos regenerating transgenic plants | Number of transgenic events (Y) | Efficiency (Y/X%) | Transmission to progeny |
|---|---|---|---|---|---|---|
| 1 | +AS | 86 | 1 | 5 | 5.8 | Yes |
| 2 | +AS | 144 | 1 | 1 | 0.7 | Yes |
| 3 | +AS | 159 | 1 | 1 | 0.6 | Yes |
| 4a | -AS | 146 | 1 | 1 | 0.7 | Yes |
| 4b | +AS | 150 | 1 | 2 | 1.3 | Yes |
| 5 | -AS | 214 | 1 | 1 | 0.5 | Yes |
| 7 | +AS | 283 | 5 | ND | ≥1.8 | ND |
| 9 | +AS | 135 | 2 | ND | ≥1.5 | ND |
| 11 | +AS | 155 | 2 | ND | ≥1.3 | ND |
| 14 | +AS | 154 | 1 | ND | ≥0.6 | ND |
| 15 | +AS | 105 | 2 | ND | ≥1.9 | ND |
| | | | | | Mean ≥1.5% | |

Table 1 : Transformation efficiencies for wheat immature embryos using seed inoculation method

| A188 | LBA 4404 (pSB131) | EHA101(pSCVSulugi) |
|---|---|---|
| Transient GUS expression Embryos GUS positive / Embryos tested (%) | 36/1345 (2.7 %) | 33/393 (8.4%) |
| Stable Transformation efficiency (Events regenerated / Embryos in selection) | 2/421 (0.5%) | |
| Hi II | | |
| Transient GUS expression Embryos GUS positive / Embryos tested (%) | 20/381 (9.2%) | 7/66 (10.6%) |
| Stable Transformation efficiency (Events regenerated / Embryos in selection) | 5 / 225 (2.2%) | |

Table 2: Efficiency of T-DNA delivery and transformation efficiency by seed inoculation of maize.

| Experiments | 1 | 2 | 3 |
|---|---|---|---|
| Embryos inoculated | 27 | 40 | 42 |
| *Agrobacterium* OD | 0.5 | 1.0 | 0.5 |
| Acetosyringone (µM) | 400 | 400 | 200 |
| Days of Co-cultivation | 2 | 5 | 5 |
| Positive Cotyledons / Cotyledons Tested | 0/24 | 2/30 | 3/38 |
| Callus induction | - | 7 | 6 |
| Positive Cotyledons / Cotyledons Tested | - | 2/35 | 0/40 |
| % of cotyledons with GUS positive spots | 0% | 6.2% | 3.8% |

Table 3 : Efficiency of T-DNA delivery by seed inoculation method in soybean

| | C58pGV2260 pBin 19 | C58pMP90 pSCV1.2GI | | | |
|---|---|---|---|---|---|
| Days after anthesis | 21 | 16 | | 13 | |
| Embryos inoculated | 107 | 17 | | 70 | |
| Callus induction | 7 | 6 | 14 | 6 | 14 |
| Positive Embryos / Embryos tested | 34/52 | 0/11 | 1/6 | 0/31 | 0/38 |
| % of explants with GUS positive spots | 65.4% | 5.9% | | 0.0% | |

Table 4 : Efficiency of T-DNA transfer to sunflower immature embryos by seed inoculation.

# INTERNATIONAL SEARCH REPORT

Int' tional Application No
PCT/EP 00/04177

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/82 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, BIOSIS, CAB Data, BIOTECHNOLOGY ABS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | EP 0 672 752 A (JAPAN TOBACCO INC)<br>20 September 1995 (1995-09-20)<br>page 5, line 33 -page 6, line 1<br>page 7, line 31 -page 8, line 1<br>claims 1-15<br>--- | 1-9,<br>12-17 |
| X | EP 0 870 838 A (INST NAC INVEST TECN AGR ALIM ;IVIA (ES))<br>14 October 1998 (1998-10-14)<br>page 4, column 2, line 45 -page 5, column 1, line 41<br>--- | 1-5,8,<br>10-17 |
| X | WO 98 56932 A (EDWARDS GLYN ALYN ;SHELL INT RESEARCH (NL))<br>17 December 1998 (1998-12-17)<br>page 3, line 13 -page 5, line 33<br>page 37 -page 56<br>---<br>-/-- | 1-5,<br>12-17 |

[X] Further documents are listed in the continuation of box C.

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| 27 July 2000 | 03/08/2000 |

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# INTERNATIONAL SEARCH REPORT

In ational Application No
PCT/EP 00/04177

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication,where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | WO 97 48814 A (MONSANTO CO)<br>24 December 1997 (1997-12-24)<br>page 15, line 11 -page 16, line 7<br>page 26, line 21 -page 36, line 9 | 1-17 |
| A | WO 86 00931 A (ATLANTIC RICHFIELD CO)<br>13 February 1986 (1986-02-13)<br>page 6, line 35 -page 7, line 34 | 1-17 |
| X | WO 99 14349 A (HELSINKI UNIVERSITY LICENSING) 25 March 1999 (1999-03-25)<br>page 2, line 5 -page 3, line 6 | 1,12-17 |

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Int[illegible]ional Application No
PCT/EP 00/04177

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
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(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
8 February 2001 (08.02.2001)

PCT

(10) International Publication Number
WO 01/09302 A2

(51) International Patent Classification[7]: C12N 15/00

(21) International Application Number: PCT/US00/20634

(22) International Filing Date: 28 July 2000 (28.07.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
09/364,254 29 July 1999 (29.07.1999) US

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(81) Designated States *(national)*: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

**Published:**
— *Without international search report and to be republished upon receipt of that report.*

*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

WO 01/09302 A2

(54) Title: A NOVEL *AGROBACTERIUM*-MEDIATED PLANT TRANSFORMATION METHOD

(57) Abstract: The present invention relates to a novel transformation system for generating transformed plants with lower copy inserts and improved transformation efficiency. In particular, the invention relates to the use of *Agrobacterium* growth inhibiting agents during the *Agrobacterium*-mediated transformation process that suppress *Agrobacterium* growth and reduce T-DNA transfer to the target plant genome.

A Novel *Agrobacterium*-mediated Plant Transformation Method

## BACKGROUND OF THE INVENTION

The present invention relates to the field of plant biotechnology. More specifically, it concerns methods of incorporating genetic components into a plant comprising a T-DNA transfer process. In particular, provided herein are systems for genetically transforming monocotyledonous plants including corn, rice, and wheat.

The method comprises novel conditions during the inoculation, co-culture, or infiltration of *Agrobacterium* with a transformable plant cell or tissue. Exemplary methods include an improved method using a bacterial growth suppressing agent during the *Agrobacterium*-mediated transformation process. The improved method can be used for introducing nucleic acids into transformable cells or tissues using a variety of selectable and/or screenable marker systems, and with a number of different plant species. The present invention also provides transgenic plants, in particular, corn, rice, and wheat. In other aspects, the invention relates to the production of stably transformed plants, gametes, and offspring from these plants.

During the past decade, it has become possible to transfer genes from a wide range of organisms to crop plants by recombinant DNA technology. This advance has provided enormous opportunities to improve plant resistance to pests, disease and herbicides, and to modify biosynthetic processes to change the quality of plant products (Knutson et al., 1992; Piorer et al., 1992). However, the availability of efficient *Agrobacterium*-mediated transformation methods suitable for high capacity production of economically important plants is limited. In particular, a novel culture system that generates reproducible transformants with a simple integration pattern of the introduced DNA into the host genome, more specifically, the integration of a low copy number (one to two copies) of the introduced DNA is needed.

There have been many methods attempted for plant transformation, but only a few methods are highly efficient. Moreover, few methods are both highly efficient and result in transformants with simple integration pattern and low copy number of the

introduced DNA . Copy number refers to the number of complete or incomplete copies of T-DNA introduced in host cell. The technologies for the introduction of DNA into cells are well known to those of skill in the art and can be divided into categories including but not limited to: (1) chemical methods (Graham and van der Eb, 1973); (2) physical methods such as microinjection (Capecchi, 1980), electroporation ( Fromm et al., 1985; U.S. Patent No. 5,384,253) and the gene gun (Christou, 1992; Fynan et al., 1993); (3) viral vectors (Clapp, 1993; Lu et al., 1993; Eglitis and Anderson, 1988);(4) receptor-mediated mechanisms (Curiel et al., 1992); and (5) *Agrobacterium*-mediated plant transformation methods.

Until recently, the methods employed for some monocot species included direct DNA transfer into isolated protoplasts and microprojectile-mediated DNA delivery (Fromm et al, 1990). The protoplast methods have been widely used in rice, where DNA is delivered to the protoplasts through liposomes, PEG, and electroporation. While a large number of transgenic plants have been recovered in several laboratories (Datta et al., 1990), the protoplast methods require the establishment of long-term embryogenic suspension cultures. Some regenerants from protoplasts are infertile and phenotypically abnormal due to the long-term suspension culture (Davey et al., 1991; Rhodes et al.,1988). U.S. patent number 5,631,152 describes a rapid and efficient microprojectile bombardment method for the transformation and regeneration of monocots and dicots.

To date, microparticle- and *Agrobacterium*-mediated gene delivery are the two most commonly used plant transformation methods. Microparticle-mediated transformation refers to the delivery of DNA coated onto microparticles that are propelled into target tissues by several methods. This method can result in transgenic events with a higher copy number, complex integration patterns, and fragmented inserts. *Agrobacterium*-mediated plant transformation can also result in transformed plants with multiple copies of inserts and complex integration patterns. A reduction in copy number can result from a decrease in the frequency of T-DNA transfer. Accordingly, novel culture conditions can be manipulated to impact the frequency of T-DNA transfer

and can produce transformation events containing the optimum number of copies of the introduced DNA.

A reproducible *Agrobacterium*-mediated method that consistently results in low copy number inserts and is applicable to a broad number of plant species is desirable for a number of reasons. For example, the presence of multiple inserts can lead to a phenomenon known as gene silencing which can occur by several mechanisms including but not limited to recombination between the multiple copies which can lead to subsequent gene loss. Also, multiple copies can cause reduced levels of expression of the gene which in turn can result in the reduction of the characteristic(s) conferred by the gene product(s). Despite the number of transformation methods available for specific plant systems, it would be advantageous to have a method of introducing genes into plants that is applicable to various crops and a variety of transformable tissues.

*Agrobacterium*-mediated transformation is achieved through the use of a genetically engineered soil bacterium belonging to the genus *Agrobacterium*. Several *Agrobacterium* species mediate the transfer of a specific DNA known as "T-DNA", that can be genetically engineered to carry any desired piece of DNA into many plant species. The major events marking the process of T-DNA mediated pathogenesis are: induction of virulence genes, processing and transfer of T-DNA. This process is the subject of many reviews (Ream, 1989; Howard and Citovsky, 1990; Kado, 1991; Hooykaas and Schilperoort, 1992; Winnans, 1992; Zambryski, 1992; Gelvin, 1993; Binns and Howitz, 1994; Hooykaas and Beijersbergen 1994; Lessl and Lanka, 1994; Zupan and Zambryski, 1995).

*Agrobacterium*-mediated genetic transformation of plants involves several steps. The first step, in which the *Agrobacterium* and plant cells are first brought into contact with each other, is generally called "inoculation". Following the inoculation step, the *Agrobacterium* and plant cells/tissues are usually grown together for a period of several hours to several days or more under conditions suitable for growth and T-DNA transfer. This step is termed "co-culture". Following co-culture and T-DNA delivery, the plant cells are often treated with bacteriocidal and-or bacteriostatic agents to kill the

*Agrobacterium*. If this is done in the absence of any selective agents to promote preferential growth of transgenic versus non-transgenic plant cells, then this is typically referred to as the "delay" step. If done in the presence of selective pressure favoring tranasgenic plant cells, then it is referred to as a "selection" step. When a "delay" is used, it is followed by one or more "selection" steps. Both the "delay" and "selection" steps typically include bacteriocidal and-or bacteriostatic agents to kill any remaining *Agrobacterium* cells because the growth of *Agrobacterium* cells is undesirable after the infection (inoculation and co-culture) process.

Although transgenic plants produced through *Agrobacterium*-mediated transformation generally contain a simple integration pattern as compared to microparticle-mediated genetic transformation, a wide variation in copy number and insertion patterns exists (Jones et al, 1987; Jorgensen et al., 1987; Deroles and Gardner, 1988). Moreover, even within a single plant genotype, different patterns of T-DNA integration are possible based on the type of explant and transformation system used (Grevelding et al., 1993). Factors that regulate T-DNA copy number are poorly understood. A reproducible, broadly applicable method to increase the efficiency of producing plants with a low copy number, and preferably a single copy of the T-DNA would be highly desirable to those practicing in the art.

Recently, monocot species have been successfully transformed via *Agrobacterium*-mediated transformation. WO 97/48814 discloses processes for producing stably transformed fertile wheat. The method describes the recovery of transgenic, wheat plants within a short period of time using a variety of explants. *Agrobacterium*-mediated transformation provides a viable alternative to bombardment methods and the method also allows more efficient molecular characterization of transgenic lines. The present invention is an improved *Agrobacterium*-mediated transformation method that relies on the control of *Agrobacterium* growth during the transformation process. More specifically, the present invention focuses on controlling *Agrobacterium* growth in the stages of *Agrobacterium*-mediated transformation during which T-DNA transfer can occur.

The major deficiencies in current plant transformation systems utilizing *Agrobacterium*-mediated methods include but are not limited to the production efficiency of the system, and transformation difficulties due to genotype or species diversity and explant limitations. WO 94/00977 describes a method for transforming monocots that depends on the use of freshly cultured immature embryos for one monocot and cultured immature embryos or callus for a different monocot. In either system, the explants must be freshly isolated, and the method is labor intensive, genotype-, and explant-limited. Other reports rely on the use of specific strains or vectors to achieve high efficiency transformation. In one report, a specific super-binary vector must be used in order to achieve high-efficiency transformation (Ishida et al., 1996).

Despite the number of transformation methods in the art, few methods have been developed that are broadly applicable to genotypes of a single crop species as well as to genotypes of other crop species. What is lacking in the art is an *Agrobacterium*-mediated plant transformation system that is efficient, reproducible, applicable to a number of plant systems, and a transformation system that effectively results in transformed plants with a simple integration pattern and a low copy number. The present invention provides novel culture conditions using one or more bacterial growth inhibiting agents during inoculation and co-culture of *Agrobacterium* with a transformable plant cell or tissue that result in increased transformation efficiencies and a low copy number of the introduced genetic component in several plant systems. The method of the present invention consistently results in desired transgenic events with a low number of inserts and reduces the need to screen hundreds of lines for identification of the optimal commercial line for breeding and introduction of improved germplasm to plant breeders, growers, and consumers. The present invention thus provides a novel improvement compared to existing *Agrobacterium*-mediated transformation methods.

## SUMMARY OF THE INVENTION

The present invention provides novel methods for the stable and efficient transformation of plants under conditions that inhibit the growth of *Agrobacterium* cells during the transformation process.

In one aspect the present invention provides a novel method of transforming a plant cell or plant tissue with *Agrobacterium* by inoculating a transformable cell or tissue containing at least one genetic component capable of being transferred to the plant cell or tissue in the presence of at least one growth inhibiting agent, co-culturing in the presence or absence of the growth inhibiting agent, selecting a transformed plant cell or tissue, and regenerating a transformed plant expressing the genetic component from the selected plant cells or tissues.

In one embodiment, the growth inhibiting agent comprises a compound containing a heavy metal such as silver, or an antibiotic such as carbenicillin, or a nucleic acid, or protein capable of inhibiting or suppressing the growth of *Agrobacterium* cells and the growth inhibiting agent is present during the inoculation step in the transformation process and not in the co-culture step.

In another embodiment, the growth inhibiting agent that is inhibitory to *Agrobacterium* cell growth is present during the inoculation and co-culture steps in the transformation process.

In another embodiment, the growth inhibiting agent that is inhibitory to *Agrobacterium* cell growth is absent during the inoculation step, but present in the co-culture step in the transformation process.

In still another embodiment the invention relates to the presence of at least one *Agrobacterium* growth inhibiting agent during the inoculation process in an amount sufficient to suppress *Agrobacterium* growth and reduce T-DNA transfer, thus favoring low copy insertions of the introduced DNA.

Still another aspect of the present invention relates to transformed plants produced by inoculating a transformable cell or tissue containing at least one genetic component capable of being transferred to the plant cell or tissue in the presence of at least one growth inhibiting agent, co-culturing in the presence or absence of the growth

inhibiting agent, selecting a transformed plant cell or tissue and regenerating a transformed plant expressing the genetic component from the selected plant cells or tissues.

Yet another aspect of the present invention relates to any seeds, or progeny of the transformed plants produced by the method of the present invention.

Further objects, advantages and aspects of the present invention will become apparent from the accompanying figures and description of the invention.

## BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a plasmid map of pMON30100

FIG. 2 is a plasmid map of pMON18365

FIG. 3 is a plasmid map of pMON25457

FIG. 4 is a plasmid map of pMON25492

FIG. 5 is a plasmid map of pMON32092

## DETAILED DESCRIPTION OF THE INVENTION

The present invention can be used with any plant species. It is particularly useful for monocot species. Particularly preferred species for practice of the present invention include corn, wheat, and rice.

The present invention provides a transgenic plant and a method for transformation of plant cells or tissues and recovery of the transformed cells or tissues into a differentiated transformed plant. To initiate a transformation process in accordance with the present invention, it is first necessary to select genetic components to be inserted into the plant cells or tissues. Genetic components can include any nucleic acid that is introduced into a plant cell or tissue using the method according to the invention. Genetic components can include non-plant DNA, plant DNA or synthetic DNA.

In a preferred embodiment, the genetic components are incorporated into a DNA composition such as a recombinant, double-stranded plasmid or vector molecule comprising at least one or more of following types of genetic components:

(a) a promoter that functions in plant cells to cause the production of an RNA sequence,

(b) a structural DNA sequence that causes the production of an RNA sequence that encodes a product of agronomic utility, and

(c) a 3' non-translated DNA sequence that functions in plant cells to cause the addition of polyadenylated nucleotides to the 3' end of the RNA sequence.

The vector may contain a number of genetic components to facilitate transformation of the plant cell or tissue and regulate expression of the desired gene(s). In one preferred embodiment, the genetic components are oriented so as to express a mRNA, that in one embodiment can be translated into a protein. The expression of a plant structural coding sequence (a gene, cDNA, synthetic DNA, or other DNA) that exists in double-stranded form involves transcription of messenger RNA (mRNA) from one strand of the DNA by RNA polymerase enzyme and subsequent processing of the mRNA primary transcript inside the nucleus. This processing involves a 3' non-translated region that adds polyadenylated nucleotides to the 3' ends of the mRNA.

Means for preparing plasmids or vectors containing the desired genetic components are well known in the art. Vectors used to transform plants and methods of making those vectors are described in U. S. Patent Nos. 4,971,908, 4,940,835, 4,769,061 and 4,757,011, the entirety of which are incorporated herein by reference. Vectors typically consist of a number of genetic components, including but not limited to regulatory elements such as promoters, leaders, introns, and terminator sequences. Regulatory elements are also referred to as cis- or trans-regulatory elements, depending on the proximity of the element to the sequences or gene(s) they control.

Transcription of DNA into mRNA is regulated by a region of DNA usually referred to as the "promoter". The promoter region contains a sequence of bases that signals RNA polymerase to associate with the DNA, and to initiate the transcription into

mRNA using one of the DNA strands as a template to make a corresponding complementary strand of RNA.

A number of promoters that are active in plant cells have been described in the literature. Such promoters would include but are not limited to the nopaline synthase (NOS) and octopine synthase (OCS) promoters that are carried on tumor-inducing plasmids of *Agrobacterium tumefaciens*, the caulimovirus promoters such as the cauliflower mosaic virus (CaMV) 19S and 35S promoters and the figwort mosaic virus (FMV) 35S promoter, the enhanced CaMV35S promoter (e35S), the light-inducible promoter from the small subunit of ribulose bisphosphate carboxylase (ssRUBISCO, a very abundant plant polypeptide). All of these promoters have been used to create various types of DNA constructs that have been expressed in plants. See, for example PCT publication WO 84/02913 (Rogers *et al.*, Monsanto, herein incorporated by reference in its entirety).

Promoter hybrids can also be constructed to enhance transcriptional activity (Hoffman, U.S. Patent No. 5,106,739), or to combine desired transcriptional activity, inducibility and tissue specificity or developmental specificity. Promoters that function in plants include but are not limited to promoters that are inducible, viral, synthetic, constitutive as described (Poszkowski *et al.*, 1989; Odell *et al.*, 1985), and temporally regulated, spatially regulated, and spatio-temporally regulated (Chau *et al.*, 1989). Other promoters that are tissue-enhanced, tissue-specific, or developmentally regulated are also known in the art and envisioned to have utility in the practice of this invention.

Promoters may be obtained from a variety of sources such as plants and plant DNA viruses and include, but are not limited to the CaMV35S and FMV35S promoters and promoters isolated from plant genes such as ssRUBISCO genes. As described below, it is preferred that the particular promoter selected should be capable of causing sufficient expression to result in the production of an effective amount of the gene product of interest.

The promoters used in the DNA constructs (i.e. chimeric/recombinant plant genes) of the present invention may be modified, if desired, to affect their control characteristics. Promoters can be derived by means of ligation with operator regions,

random or controlled mutagenesis. etc. Furthermore. the promoters may be altered to contain multiple "enhancer sequences" to assist in elevating gene expression. Examples of such enhancer sequences have been reported by Kay *et al.* (1987).

The mRNA produced by a DNA construct of the present invention may also contains a 5' non-translated leader sequence. This sequence can be derived from the promoter selected to express the gene, and can be specifically modified so as to increase translation of the mRNA. The 5' non-translated regions can also be obtained from viral RNAs, from suitable eukaryotic genes, or from a synthetic gene sequence (Griffiths, *et al.*, 1993) Such "enhancer" sequences may be desirable to increase or alter the translational efficiency of the resultant mRNA. The present invention is not limited to constructs wherein the non-translated region is derived from both the 5' non-translated sequence that accompanies the promoter sequence. Rather, the non-translated leader sequence can be derived from unrelated promoters or genes. (see, for example U. S. Patent 5,362865). Other genetic components that serve to enhance expression or affect transcription or translational of a gene are also envisioned as genetic components.

The 3' non-translated region of the chimeric constructs should contain a transcriptional terminator, or an element having equivalent function, and a polyadenylation signal that functions in plants to cause the addition of polyadenylated nucleotides to the 3' end of the RNA. Examples of suitable 3' regions are (1) the 3' transcribed, non-translated regions containing the polyadenylation signal of *Agrobacterium* tumor-inducing (Ti) plasmid genes, such as the nopaline synthase (NOS) gene, and (2) plant genes such as the soybean storage protein genes and the small subunit of the ribulose-1,5-bisphosphate carboxylase (ssRUBISCO) gene. An example of a preferred 3' region is that from the ssRUBISCO E9 gene from pea (Fischhoff *et al.*, European Patent Application 0385 962, herein incorporated by reference in its entirety).

Typically, DNA sequences located a few hundred base pairs downstream of the polyadenylation site serve to terminate transcription. The DNA sequences are referred to herein as transcription-termination regions. The regions are required for efficient polyadenylation of transcribed messenger RNA (mRNA) and are known as 3' non-

translated regions. RNA polymerase transcribes a coding DNA sequence through a site where polyadenylation occurs.

In one preferred embodiment, the vector contains a selectable, screenable, or scoreable marker gene. These genetic components are also referred to herein as functional genetic components, as they produce a product that serves a function in the identification of a transformed plant, or a product of agronomic utility. The DNA that serves as a selection device functions in a regenerable plant tissue to produce a compound that would confer upon the plant tissue resistance to an otherwise toxic compound. Genes of interest for use as a selectable, screenable, or scorable marker would include but are not limited to GUS, green fluorescent protein (GFP), luciferase (LUX), antibiotic or herbicide tolerance genes. Examples of transposons and associated antibiotic resistance genes include the transposons Tns (*bla*), Tn5 (*npt*II), Tn7 (*dhfr*), penicillins, kanamycin (and neomycin, G418, bleomycin); methotrexate (and trimethoprim); chloramphenicol; kanamycin and tetracycline.

Characteristics useful for selectable markers in plants have been outlined in a report on the use of microorganisms (Advisory Committee on Novel Foods and Processes, July 1994). These include:

i ) stringent selection with minimum number of nontransformed tissues;

ii ) large numbers of independent transformation events with no significant interference with the regeneration;

iii ) application to a large number of species; and

iv) availability of an assay to score the tissues for presence of the marker.

As mentioned, several antibiotic resistance markers satisfy these criteria, including those resistant to kanamycin (*nptII*), hygromycin B (*aph IV*) and gentamycin (*aac3* and *aac*C4).

A number of selectable marker genes are known in the art and can be used in the present invention (see for example Wilmink and Dons, 1993). Particularly preferred selectable marker genes for use in the present invention would include genes that confer resistance to compounds such as antibiotics like kanamycin (Dekeyser et al., 1989), and herbicides like glyphosate (Della-Cioppa et al., 1987). Other selection devices can also

be implemented including but not limited to tolerance to phosphinothricin, bialaphos, and positive selection mechanisms (Joersbo et al., 1998) and would still fall within the scope of the present invention.

The present invention can be used with any suitable plant transformation plasmid or vector containing a selectable or screenable marker and associated regulatory elements as described, along with one or more nucleic acids expressed in a manner sufficient to confer a particular desirable trait. Examples of suitable structural genes of agronomic interest envisioned by the present invention would include but are not limited to genes for insect or pest tolerance, herbicide tolerance, genes for quality improvements such as yield, nutritional enhancements, environmental or stress tolerances, or any desirable changes in plant physiology, growth, development, morphology or plant product(s).

Alternatively, the DNA coding sequences can effect these phenotypes by encoding a non-translatable RNA molecule that causes the targeted inhibition of expression of an endogenous gene, for example via antisense- or cosuppression-mediated mechanisms (see, for example, Bird et al., 1991). The RNA could also be a catalytic RNA molecule (i.e., a ribozyme) engineered to cleave a desired endogenous mRNA product (see for example, Gibson and Shillitoe, 1997). Thus, any gene that produces a protein or mRNA that expresses a phenotype or morphology change of interest are useful for the practice of the present invention.

Exemplary nucleic acids that may be introduced by the methods encompassed by the present invention include for example, DNA sequences or genes from another species, or even genes or sequences that originate with or are present in the same species, but are incorporated into recipient cells by genetic engineering methods rather than classical reproduction or breeding techniques. However, the term exogenous is also intended to refer to genes that are not normally present in the cell being transformed, or perhaps simply not present in the form, structure, etc., as found in the transforming DNA segment or gene, or genes that are normally present yet that one desires, *e.g.*, to have over-expressed. Thus, the term "exogenous" gene or DNA is intended to refer to any gene or DNA segment that is introduced into a recipient cell.

regardless of whether a similar gene may already be present in such a cell. The type of DNA included in the exogenous DNA can include DNA that is already present in the plant cell, DNA from another plant, DNA from a different organism, or a DNA generated externally, such as a DNA sequence containing an antisense message of a gene, or a DNA sequence encoding a synthetic or modified version of a gene.

In light of this disclosure, numerous other possible selectable and/or screenable marker genes, regulatory elements, and other sequences of interest will be apparent to those of skill in the art. Therefore, the foregoing discussion is intended to be exemplary rather than exhaustive.

After the construction of the plant transformation vector or construct, said nucleic acid molecule, prepared as a DNA composition *in vitro*, is introduced into a suitable host such as *E. coli* and mated into another suitable host such as *Agrobacterium*, or directly transformed into competent *Agrobacterium*. These techniques are well-known to those of skill in the art and have been described for a number of plant systems including soybean, cotton, and wheat (See, for example U. S. Patent Nos. 5,569834, 5,159135, and WO 97/48814 herein incorporated by reference in their entirety).

The present invention encompasses the use of bacterial strains to introduce one or more genetic components into plants. Those of skill in the art would recognize the utility of *Agrobacterium*-mediated transformation methods. A number of wild-type and disarmed strains of *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* harboring Ti or Ri plasmids can be used for gene transfer into plants. Preferably, the *Agrobacterium* hosts contain disarmed Ti and Ri plasmids that do not contain the oncogenes which cause tumorigenesis or rhizogenesis, respectfully, which are used as the vectors and contain the genes of interest that are subsequently introduced into plants. Preferred strains would include but are not limited to *Agrobacterium tumefaciens* strain C58, a nopaline-type strain that is used to mediate the transfer of DNA into a plant cell, octopine-type strains such as LBA4404 or succinamopine-type strains e.g., EHA101 or EHA105. The use of these strains for plant transformation has been reported and the methods are familiar to those of skill in the art.

The present invention can be used in any plant transformation system. Examples of suitable plant targets for the practice of the present invention would include but are not limited to alfalfa, barley, canola, corn, cotton, oats, potato, rice, rye, soybean, sugarbeet, sunflower, sorghum, and wheat. Particularly preferred dicotyledonous targets would include soybean, cotton, canola, or sunflower. Particularly preferred monocotyledonous targets would include cereals such as corn, wheat, and rice.

The present invention can be used with any transformable cell or tissue. By transformable as used herein is meant a cell or tissue that is capable of further propagation to give rise to a plant. Those of skill in the art recognize that a number of plant cells or tissues are transformable in which after insertion of exogenous DNA and appropriate culture conditions the plant cells or tissues can form into a differentiated plant. Tissue suitable for these purposes can include but is not limited to immature embryos, scutellar tissue, suspension cell cultures, callus tissue, hypocotyl tissue, cotyledons, roots, and leaves. Preferred explants for dicots include but are not limited to leaf, root, cotyledon, callus, inflorescence, hypocotyl, and stem. Preferred explants for monocots include but are not limited to immature embryos, embryogenic calli, immature inflorescence, root, shoot meristem, node, nodal explants and cell suspensions.

The explants can be from a single genotype or from a combination of genotypes. In a preferred embodiment, superior explants from plant hybrids can be used as explants. For example, a fast-growing cell line with a high culture response (higher frequency of embryogenic callus formation, growth rate, plant regeneration frequency, etc.) can be generated using hybrid embryos containing several genotypes. In a preferred embodiment an F1 hybrid or first generation offspring of cross-breeding can be used as a donor plant and crossed with another genotype. For example, Pa91 which is an inbred line is crossed with a second inbred line such as H99 and the resulting F1 hybrid plant is crossed with inbred A188. Those of skill in the art are aware that heterosis also referred to herein as "hybrid vigor" occurs when two inbreds are crossed. The present invention thus encompasses the use of an explant resulting from a three-way or "triple hybrid" cross, wherein at least one or more of the inbreds is highly

regenerable and transformable, and the transformation and regeneration frequency of the triple hybrid explant exceeds the frequencies of the inbreds individually. Other tissues are also envisioned to have utility in the practice of the present invention.

In a preferred embodiment of the present invention, immature embryos (IEs) of corn, rice, and wheat are used as explants for *Agrobacterium*-mediated transformation. In wheat for example, immature embryos may be isolated from wheat spikelets. The isolation of wheat immature embryos is also described by Weeks et al., (1993) and Vasil et al., (1993). Similarly, corn ears are harvested approximately 8-16 days after pollination and used as a source of immature embryos. In rice, immature caryopses are collected from plants after anthesis and immature embryos isolated from these caryopses are used as explants. The present invention thus encompasses the use of freshly isolated embryos as described. In another embodiment a suspension cell culture can be used as suitable plant material for transformation. In another embodiment a precultured tissue is used as the target plant material for transformation. By precultured as used herein is meant culturing the cells or tissues in an appropriate medium to support plant tissue growth prior to inoculation with *Agrobacterium*. The preculture of the transformable cells or tissue prior to *Agrobacterium* inoculation can occur for any length of time, for example from one day to seven days. Preferably the preculture period is less than seven days. More preferably the preculture period is three days or less. Even more preferably, the preculture of the transformable cells or tissues is from 18 - 28 hours.

Any suitable plant culture medium can be used for the preculture. Examples of suitable media for preculture would include but are not limited to MS-based media (Murashige and Skoog, 1962) or N6-based media (Chu et al., 1975) supplemented with additional plant growth regulators including but not limited to auxins such as picloram (4-amino-3,5,6-trichloropicolinic acid), 2,4-D (2,4-dichlorophenoxyacetic acid) and dicamba (3,6-dichloroanisic acid), cytokinins such as BAP (6-benzylaminopurine ) and kinetin, and gibberellins. Other media additives can include but are not limited to amino acids, macroelements, iron, microelements, vitamins and organics, carbohydrates, undefined media components such as casein hydrolysates, an appropriate gelling agent such as a form of agar, such as a low melting point agarose or

Gelrite if desired. Those of skill in the art are familiar with the variety of tissue culture media, which when supplemented appropriately, support plant tissue growth and development and are suitable for plant transformation and regeneration. These tissue culture media can either be purchased as a commercial preparation, or custom prepared and modified. Examples of such media would include but are not limited to Murashige and Skoog (Murashige and Skoog, 1962) , N6 (Chu et al., 1975), Linsmaier and Skoog (Linsmaier and Skoog, 1965), Uchimiya and Murashige (Uchimiya and Murashige, 1974), Gamborg's media (Gamborg et al., 1968), D medium (Duncan et al., 1985), McCown's Woody plant media (McCown and Loyd, 1981), Nitsch and Nitsch (Nitsch and Nitsch, 1969), and Schenk and Hildebrandt (Schenk and Hildebrandt, 1972) or derivations of these media supplemented accordingly. Those of skill in the art are aware that media and media supplements such as nutrients and growth regulators for use in transformation and regeneration and other culture conditions such as light intensity during incubation, pH, and incubation temperatures that can be optimized for the particular target crop of interest.

Once the transformable plant tissue is isolated, the next step of the method is introducing the genetic components into the plant tissue. This process is also referred to herein as "transformation." The plant cells are transformed and each independently transformed plant cell is selected. The independent transformants are referred to as transgenic events. A number of methods have been reported and can be used to insert genetic components into transformable plant tissue.

Methods for transforming dicots, primarily by use *of Agrobacterium tumefaciens*, and obtaining transgenic plants have been published for a number of crops including cotton (U.S. Patent No. 5,064,863; U.S. Patent No. 5,159,135; U. S. Patent No. 5,518,908, WO 97/43430), soybean (U. S. Patent No. 5,569,834; U. S. Patent No. 5,416,011; McCabe et al. (1988); Christou et al. (1988), *Brassica* (U. S. Patent No. 5,463,174), peanut (Cheng et al. (1996); De Kathen and Jacobsen (1990)).

Transformation of monocots using electroporation, particle bombardment, and *Agrobacterium* have also been reported. Transformation and plant regeneration have been achieved in asparagus (Bytebier et al. (1987)), barley (Wan and Lemaux (1994),

Tingay et al., (1997)' maize (Rhodes et al. (1988); Ishida et al. (1996); Gordon-Kamm et al. (1990); Fromm et al. (1990); Koziel et al. (1993); Armstrong et al. (1995), oat (Somers et al. (1992)), rice (Toriyama et al. (1988); Zhang and Wu (1988); Zhang et al.